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(54) Title: METHODS FOR TREATING CANCER AND FOR MEDIATING CHEMOTAXIS OF DENDRITIC CELLS

(57) Abstract

The invention is based on the novel observation, reported herein, that SLC inhibits the growth of tumors *in vivo* and is a potent chemokine for Dendritic Cells (DC). Thus, the invention provides a method of treating cancer or hyperproliferative disorder in a mammalian subject, comprising: administering a therapeutically effective amount of an SLC to said subject. SLCs useful in the methods of the invention include SLC polypeptides, variants and fragments and related nucleic acids. The methods of the invention are useful in the treatment of cancer and hyperproliferative disorders. In addition, the invention provides methods for modulating dendritic cell function in a mammal, said method comprising administering a therapeutically effective amount of an SLC agent to said mammal, said agent selected from the group consisting of: SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding SLC polypeptides, variants and fragments, anti-SLC antibodies and ligands for the CCR7 receptor. Such methods can be used to increase or decrease an immune response, particularly a primary immune response, in a mammal. The methods are particularly useful in the prevention of graft rejection and in the treatment of autoimmune disease. Alternatively, the methods may be used to enhance an immune response.

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METHODS FOR TREATING CANCER AND FOR MEDIATING CHEMOTAXIS OF DENDRITIC CELLS

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FIELD OF THE INVENTION

The invention relates to chemokines useful in the treatment of cancer and hyperproliferative cell growth, and for the modulation of immune responses.

BACKGROUND OF THE INVENTION

10 Chemokines constitute a group of over 30 small (8-12kDa), heparin-binding cytokines with common structural features that mediate leukocytes migration into sites of inflammation and immune responses. In addition, some chemokines have been shown to have other biological activity such as the modulation of hemopoiesis, angiogenesis, suppression of apoptosis, and HIV-1 absorption. Most chemokines 15 contain four conserved cysteine residues and are grouped into four major subfamilies, CXC, CC, C and CX₃C, based on the position of their conserved cysteine residues. In CC chemokines, the two amino terminal cysteine residues are contiguous. CC chemokines are generally chemotactic to monocytes, eosinophils, basophils, and/or lymphocytes with variable selectivity.

20 A novel human CC chemokine termed secondary lymphoid chemokine (SLC), also known as 6Ckine, Exodus-2, and thymus-derived chemotactic agent 4 (TCA4), has recently been identified. SLC differs from most other CC chemokines in structure, chromosomal localization, pattern of tissue expression, and receptor usage. In contrast to other CC chemokines, SLC has been reported to be chemotactic to lymphocytes, with 25 preferential activity toward naïve T cells, but is not chemotactic to monocytes or neutrophils. Nagira *et al.* (1997) *J. Biol. Chem.* 272:19518-19524; and Gunn *et al.* (1998) *Proc Natl Acad Sci USA* 95:258. In addition, SLC was recently shown to have angiostatic activity in a rat corneal micropocket model of angiogenesis. Soto *et al.* (1998) *Proc Natl Acad Sci USA* 95:8205. SLC is primarily expressed in lymphoid 30 tissues such as lymph nodes, Peyer's patches, appendix, spleen and the lymphatic

endothelium of multiple organs, and also in HEV endothelial cells. It has been postulated that SLC plays a major role in the recruitment of lymphocytes into secondary lymphoid organs, such as Peyer's patches and lymph nodes. See Pachynski *et al.* (1998) *J. Immunol.* 161:952-956.

5 Various applications of cytokines for the treatment of cancer and hyperproliferative disorders have been attempted. For example, the introduction of autologous tumor cells transfected with cytokine genes such as IL-2 or GM-CSF has induced protective T-cell responses to tumors. However, before the instant invention, there has been no attempt to use SLC in the treatment of cancer.

10 In one aspect, the invention described herein relates to novel methods of using the cytokine SLC in the treatment of cancer and hyperproliferative disorders.

In another aspect, the methods of the invention relate to novel use of SLC in the modulation of primary immune responses. Every immune response is a complex and intricately regulated sequence of events involving several cell types. It is triggered 15 when an antigen enters the body and encounters a specialized class of cells called antigen-presenting cells (APCs). These APCs capture a minute amount of the antigen and display it in a form that can be recognized by antigen-specific helper T lymphocytes. The helper T (T_H) cells become activated and, in turn, promote the activation of other classes of lymphocytes, such as B cells or cytotoxic T cells. The 20 activated lymphocytes then proliferate and carry out their specific effector functions, which, in many cases successfully activate or eliminate the antigen. At each stage in the process, the lymphocytes and APCs communicate with one another through direct contact or by secreting regulatory cytokines, activation molecules, and can indirectly communicate through other cells.

25 The contact between an APC and an antigen-specific T_H cell also has effects on the APC. One of the most important effects is that the APC may begin to release a cytokine called interleukin-1 (IL-1). IL-1 increases surface expression of class II MHC proteins and of various adhesion molecules, thereby strengthening binding of the T_H

cell, and enhancing antigen presentation. The T_H cell is activated to release cytokines and other growth and differentiation factors.

The term "antigen-presenting cell (APC)" refers to cells that constitutively express class II MHC molecules and present stimulatory antigens to T_H cells. There are 5 three major classes of cells that function as APCs. These classes are macrophages, dendritic cells, and B cells. Dendritic cells are most potent among antigen presenting cells and are believed to be indispensable to the initiation of a primary immune response (Lanzavecchia (1993) *Science* 260:937; and Grabbe *et al.* (1995) *Immunol Today* 16:117).

10 Dendritic cells (DC) are dedicated antigen-presenting cells that stimulate T cell-dependent immune responses (Banchereau *et al.* (1998) *Nature* 392, 6673:245; Hart, D.N.J. (1997) *Blood* 90, 9:3245). This process involves the capture and processing of antigens by DC in the periphery, their migration to regional lymph nodes *via* the lymphatics and the presentation of the processed antigens to T cells. Bacterial products 15 such as LPS and inflammatory signals such as TNF α and IL-1 have been shown to induce the maturation of DC, which is characterized by an increased surface expression of MHC class I and class II proteins, upregulation of T cell costimulatory molecules such as CD80, CD86, and CD40, as well as enhanced ability to stimulate T cells (Banchereau *et al.* (1998) *Nature* 392, 6673:245). Chemokines such as C5a, fMLP 20 SDF-1, MCP-3, MCP-4, RANTES, MIP1 α , MIP1 β , MIP-5, and MDC have been reported to induce the migration of immature DC *in vitro* (Sozzani *et al.* (1995) *J. Immunol.* 155, 7:3292; Morelli *et al.* (1996) *Immunology* 89, 1:126; Xu *et al.* (1996) *J. Leukoc. Biol.* 60, 3:365; Sozzani *et al.* (1997) *J. Immunol.* 159, 4:1993; Godiska *et al.* (1997) *J. Exp. Med.* 185, 9:1595).

25 The growth of clinical immunology has uncovered increasing numbers of diseases that are due to aberrant immune responses. For example, more than 40 diseases thought to result from aberrant immune responses are potentially treatable by agents capable of inhibiting immune responses. Accordingly, methods and therapies for

the modulation the immune responses will be useful in the treatment of these diseases and in preventing transplant rejection.

Thus, in a second aspect, the present invention provides methods for the modulation of immune responses through the manipulation of dendritic cells (DC).

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SUMMARY OF THE INVENTION

The invention is based on the novel observation, reported herein, that SLC inhibits the growth of tumors *in vivo* and is a potent chemokine for Dendritic Cells (DC). Thus, the invention provides a method of treating cancer or hyperproliferative disorder in a mammalian subject, comprising: administering a therapeutically effective amount of an SLC to said subject. SLCs useful in the methods of the invention include SLC polypeptides, variants and fragments and related nucleic acids. The methods of the invention are useful in the treatment of cancer and hyperproliferative disorders.

In addition, the invention provides methods for modulating dendritic cell function in a mammal, said method comprising administering a therapeutically effective amount of an SLC agent to said mammal, said agent selected from the group consisting of: SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding SLC polypeptides, variants and fragments, anti-SLC antibodies and ligands for the CCR7 receptor. Such methods can be used to increase or decrease an immune response, particularly a primary immune response, in a mammal. The methods are particularly useful in the prevention of graft rejection and in the treatment of autoimmune disease. Alternatively, the methods may be used to enhance an immune response.

25

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. cDNA and amino acid sequences of human SLC (SEQ ID NO:1 and SEQ ID NO:2, respectively). The arrow between amino acid residues 23 and 24 indicates the predicted cleavage site of the signal sequence.

Figure 2. cDNA and amino acid sequences of mouse SLC (SEQ ID NO:3 and SEQ ID NO:4, respectively).

Figure 3. SLC and ELC but not RANTES induced chemotaxis of mature DC. (a-b) Migration of human monocyte-derived DC matured with MCM (black bars) or TNF α (striped bars) compared to untreated immature cells (white bars), in response to the indicated concentrations (in ng/ml) of (a) SLC or (b) ELC. The percent migration (+/- SE) is based on the number of cells added to the top chamber. (c) Migration of MCM-treated mature DC or untreated (immature) DC in response to the indicated concentrations (in ng/ml) of RANTES. Chemotactic index is the ratio of the number of cells in the bottom chamber in the test case to the number of cells in the absence of the chemokine. (d) Preincubation with 100 ng/ml pertussis toxin (PT) inhibited SLC-induced migration of MCM-treated mature DC. These results are representative of at least three independent experiments

Figure 4. Calcium mobilization in MCM-treated mature DC induced by SLC and ELC. DC (a) without or (b-e) with MCM-treatment were loaded with Fluo-3AM. The chemokines were added at the times indicated by the arrows and the calcium responses analyzed by flow cytometry. (a-c) Calcium responses to 500 ng/ml of SLC, ELC or RANTES. (d-e) Dose dependent calcium responses induced by 500, 150, 50, 15, 5 and 1.5 ng/ml of (d) SLC or (e) ELC. (f-g) MCM-treated DC were loaded with Fluo-3AM and calcium mobilization was analyzed by flow cytometry. Concentration of the chemokines added were: (f) 100 ng/ml SLC, 500 ng/ml ELC, (g) 500 ng/ml ELC, 100 ng/ml SLC. These results are representative of two or more independent experiments.

Figure 5. Chemokine receptor expression profile in DC. Total RNA was prepared from DC after 8 days of culture, either untreated (-) or treated with MCM (+) from days 5-8. First strand cDNA were synthesized and used in semi-quantitative PCR analysis. PCR products from 30 cycles of amplification were visualized on an ethidium-bromide stained 2% agarose gel. Molecular weight markers (in kb) are shown to the left of the gels. The annealing temperatures for all PCR reactions were 55°C

except for CCR5, CCR6, CCR9, CXCR3, CXCR5, CX₃CR1, which were at 60°C. These results are representative of 7 or more experiments using three independent pairs of cDNA.

Figure 6. Effect of peritumor administration of SLC on survival of mice
5 injected subcutaneously with B16-BL6 mouse melanoma cells.

Figure 7. Effect of peritumor administration of SLC on primary tumor growth of B16-BL6 mouse melanoma cells injected subcutaneously into mice.

Figure 8. Effect of peritumor administration of SLC on primary tumor growth of B16-BL6 mouse melanoma cells at Day 13.

10 Figure 9. Effect of SLC in combination with IL-2 on subcutaneous B16-BL6 tumor growth in mice.

DETAILED DESCRIPTION OF THE INVENTION

15 The invention is based on the novel discoveries disclosed herein that Secondary Lymphoid-Tissue Chemokine (SLC) inhibits the growth of tumors and is chemotactic for mature Dendritic Cells (DC). While the invention is not limited by the mechanism proposed herein, it is believed that SLC inhibits tumor growth and hyperproliferative disorder by mediating immunological responses and by inhibiting angiogenesis. In 20 particular, SLC acts as a chemoattractant for DC, naïve T cells and activated T cells. While it has previously been shown that SLC inhibits bFGF or VEGF-induced angiogenesis in a rat cornea model, this is the first report demonstrating the antitumor activity of SLC *in vivo*.

25 Thus, in one aspect, the invention is directed to a method of treating cancer or hyperproliferative cell growth in a mammalian subject, comprising: administering a therapeutically effective amount of an SLC to said subject.

By "SLC polypeptide or protein" is meant a Secondary Lymphoid-Tissue Chemokine. SLC includes naturally occurring mammalian SLCs, and variants and fragments thereof, as defined below. Preferably the SLC is of human or mouse origin.

Most preferably the SLC is human SLC. Human SLC has been cloned and sequenced. Nagira *et al.* (1997) *J Biol Chem* 272:19518; the contents of which are incorporated by reference. The cDNA and amino acid sequences of human SLC are shown in Figure 1 (SEQ ID NO:1 and SEQ ID NO:2, respectively). The signal sequence of human SLC is 5 cleaved to yield the mature form, representing residues 24 to 134 of SEQ ID NO:2 (SEQ ID NO:5). Mouse SLC has also been cloned and sequenced. Hromas *et al.* (1997) *J Immunol* 159:2554; Hedrick *et al.* (1997) *J Immunol* 159:1589; and Tanabe *et al.* (1997) *J Immunol* 159:5671; the contents of which are incorporated herein by reference. The cDNA and amino acid sequences of mouse SLC are shown in Figure 2 10 (SEQ ID NO:3 and SEQ ID NO:4, respectively). The signal sequence of mouse SLC is cleaved to yield the mature form, representing residues 24 to 133 of SEQ ID NO:2 (SEQ ID NO:6).

The term "SLC polypeptide" also includes variants and fragments of the SLCs 15 of SEQ ID NO:5 and 6. For the purposes of the invention, SLC polypeptides and variants will have at least 70% sequence identity with the polypeptide of SEQ ID NO:4 or SEQ ID NO:5. Methods for determining the percent sequence identity are discussed below.

"SLC polypeptide variant" refers to a polypeptide derived from the SLC protein 20 of SEQ ID NO:5 or 6 by deletion or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants include mutants, fragments, allelic variants, homologous orthologs, and fusions of native SLC 25 polypeptide sequences. SLC polypeptides, SLC polypeptide variants and SLC fragments useful in the methods of the invention may be modified by glycosylation, phosphorylation, substitution of non-natural amino acid analogs and the like.

For the purposes of the invention, SLC variants will have at least 70%, generally at least 75%, 80%, 85%, preferably about 90% to 95% or more, and more preferably about 98% or more sequence identity to the amino acid sequence of SEQ ID NO:5 or 6.

A variant of the SLC proteins useful in the methods of the invention may differ from the polypeptides of SEQ ID NO:5 or 6 by as few as 1-33 amino acid residues, as few as 1-30, as few as 1-20, as few as 1-15, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

5 Methods for calculating identity and similarity are known in the art. See, for example, *Computer Analysis of Sequence Data, Part 1*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). In general, to determine the
10 percent identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent identity = number of identical positions/total number of positions (e.g., overlapping positions) x 100). For example, by a polypeptide having an amino acid sequence at least 95%
15 "identical" to a reference amino acid sequence is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or
20 anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The determination of percent identity between two sequences can be
accomplished using a mathematical algorithm. For the purposes of the invention, the
25 percentage sequence identity between two polypeptide sequences is determined using the BESTFIT computer program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711), using the default settings. When using BESTFIT to determine whether a particular sequence is, for instance, 95% identical to a reference sequence

according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

5 The SLC variants useful in the methods of the invention may be obtained by amino acid substitutions, deletions, truncations, and insertions. Preferred SLC polypeptide variants have one or more conservative amino acid substitutions of the polypeptide of SEQ ID NO:5 or 6. For example, conservative amino acid substitutions may be made at one or more amino acid residues. Preferably, substitutions are made at 10 nonessential amino acid residues.

A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of an SLC protein (e.g., the sequence of SEQ ID NO:5 or 6) without altering the one of the biological activities, whereas an "essential" amino acid residue is required for a given biological activity.

15 A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, 20 asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). See, for example, Bowie *et al.* (1990) *Science* 247:1306, herein incorporated by reference. Preferably, such 25 substitutions would not be made for conserved cysteine residues, such as the amino terminal contiguous cysteine residues.

The SLC variants useful in the methods of the invention may be isolated from naturally occurring variants, isolated after mutagenesis or recombinant manipulation, or be synthetically produced. Naturally occurring allelic variants can be identified with

the use of well-known molecular biology techniques, such as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Methods for such manipulations are generally known in the art. In addition, variants of the SLC proteins can be prepared by mutagenesis or recombinant manipulations. Methods for 5 mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions 10 that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.). herein incorporated by reference.

Thus, the polypeptides useful in the methods of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such 15 variants will continue to possess the desired SLC activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

By "SLC polypeptide fragment" is intended a portion of the amino acid 20 sequence of SEQ ID NO:5 or 6. As used herein, SLC polypeptide fragments will retain at least 30% of the dendritic cell chemoattractant activity, the anti-tumor activity, or the angiostatic activity of the polypeptide of SEQ ID NO:5 or 6. In addition, for the purposes of the invention, an SLC fragment will comprise at least 10 contiguous amino acid residues of polypeptide of SEQ ID NO:5 or 6. Thus, SLC polypeptide fragments 25 may range from at least 10 contiguous amino acid residues of SEQ ID NO:5 or 6, about 15, about 20, about 25, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, and up to 110-11 contiguous amino acid residues of the polypeptide of SEQ ID NO:5 or 6.

Variant SLC proteins and SLC polypeptide fragments useful in the methods of the present invention must possess SLC biological activity. Specifically, they must possess the desired biological activity of the native protein, that is, the dendritic cell-chemoattractant activity, angiostatic activity or anti-tumor activity as described herein.

5 For the purposes of the invention, a "SLC variant" will exhibit at least 30% of a dendritic cell-chemoattractant activity, tumor inhibitory activity or angiostatic activity of the polypeptide of SEQ ID NO:5 or 6. More typically, variants exhibit more than 60% of at least one of these activities; even more typically, variants exhibit more than 80% of at least one of these activities.

10 The deletions, insertions, and substitutions of the protein sequences useful herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the dendritic cell chemoattractant 15 activity can be evaluated by standard chemotaxis assays, such as the one described in Example 2, below, and by other chemotaxis assays known to those skilled in the art. See, for example, Rubbert *et al.*, (1998) *J Immunol* 160:3933 and Nagira *et al.* (1997) *J Biol Chem* 272:19518; herein incorporated by reference. Similarly, angiostatic activity can be measured in egg chorioallantoic membrane or rodent cornea models of 20 angiogenesis. See, for example, Soto *et al.* (1998) *Proc Natl Acad Sci USA* 95:8205, the contents of which are incorporated herein by reference. Anti-tumor activity may be determined using implantation of tumor cells in non-human mammalian subjects, as described in Examples 6 and 7.

25 Amino acids which are involved in binding of the SLC to dendritic cells are essential for chemotactic activity to SLC to dendritic cells. Such amino acids can be identified by methods known in the art. Such methods include alanine-scanning mutagenesis, molecular evolution (Crameri *et al.* (1996) *Nat. Biotechnol.* 14(3):315-319; Crameri *et al.* (1998) *Nature* 395:288-291; Patten *et al.* (1997) *Curr. Opin. Biotechnol.* 8:724-733; Stemmer, W.P. (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-51;

Stemmer, W.P. (1994) *Nature* 370:389-391), or site-directed mutagenesis. See, Cunningham *et al.* (1989) *Science* 244:1081. Resulting mutants can be tested for biological activity. Sites critical for binding can be determined by structural analysis such as crystallization, photoaffinity labeling, or nuclear magnetic resonance. See, 5 deVos *et al.* (1992) *Science* 255:306 and Smith *et al.* (1992) *J. Mol. Biol.* 224:899.

The methods of the invention are useful in the treatment of hyperproliferative disorders and cancers, and are particularly useful in the treatment of solid tumors. Types of solid tumors that may be treated according to the methods of the invention include, but are not limited to, melanoma, breast cancer, tumors of the head and neck, 10 ovarian cancer, endometrial cancer, urinary tract cancers, stomach cancer, testicular cancer, prostate cancer, lung cancer, bladder cancer, pancreatic cancer, bone cancer, liver cancer, colon cancer, rectal cancer, metastases of the above, and metastases of unknown primary origin. Hyperproliferative disorders that may be treated by the methods of the invention include, but are not limited to, prostatic hyperplasia, 15 proliferative breast diseases, proliferative retinopathy and pigmented skin lesions.

Angiogenesis, or development of new blood vessels, is implicated in a host of diseases including tumorigenesis, metastasis and tumor growth, retinopathies, neovascular ocular disorders, and postangioplasty or postatherectomy restenosis (Bicknell *et al.* (1996) *Curr. Opin. Oncol.* 8:60-65; Gariano *et al.* (1996) *Survey Ophthalmol.* 40:481-490; and Wilcox, J.N. (1993) *Am. J. Cardiol.* 72:88E-95E). The 20 methods of the invention are useful in suppressing angiogenesis particularly angiogenesis involved in cancer, tumorigenesis, metastasis and tumor growth. Thus, the invention provides a method for the treatment of cancer or a hyperproliferative disorder in a mammalian subject, comprising: administering a therapeutically effective 25 amount of an SLC to said subject.

The SLC may be administered directly by introducing a SLC polypeptide, SLC variant or SLC fragment into or onto the subject. Alternatively, the SLC may be produced in situ following the administration of a polynucleotide encoding a SLC polypeptide, SLC variant or SLC fragment may be introduced into the subject.

Methods of introducing and expressing polynucleotides in mammalian subjects are known to those skilled in the art. Such methods include, but are not limited to gene therapy methods. Thus, in one embodiment, the administration of SLC comprises introducing a SLC polynucleotide into a mammal.

5 SLC polynucleotides useful in the methods of the invention include, but are not limited to:

- a) the polynucleotide of SEQ ID NO:1 or 3;
- b) a polynucleotide having at least 70% sequence identity with the polynucleotide of SEQ ID NO:1 or 3;
- 10 c) a polynucleotide having at least 70% sequence identity with the coding region of the polynucleotide of SEQ ID NO:1 or 3.
- d) a polynucleotide encoding the polypeptide of SEQ ID NO:5 or 6;
- e) a polynucleotide encoding a polypeptide having at least 70% sequence identity to SEQ ID NO:5 or 6, wherein said polypeptide has at least 30% of the 15 angiostatic activity, tumor-inhibiting activity, and/or the dendritic cell-chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and
- f) a polynucleotide encoding a fragment of the polypeptide of SEQ ID NO:5 or 6, wherein said fragment has at least 30% of the angiostatic activity, tumor-inhibiting activity, and/or the dendritic cell-chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and said fragment comprises at least 20 contiguous amino acid residues of SEQ ID NO:5 or 6.

In yet another aspect, the invention comprises a method for modulating dendritic cell function in a mammal, said method comprising administering a therapeutically effective amount of an SLC agent to said mammal, said agent selected from the group 25 consisting of: SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding SLC polypeptides, variants and fragments, anti-SLC antibodies and ligands for the CCR7 receptor.

The present invention recognizes that secondary lymphoid chemokine (SLC) is chemotactic to mature DC as well as other immune cells including T cells. While the

invention is not bound by any mechanism, it is believed that SLC is involved in binding and guiding the migration of DC and other cells to secondary lymphoid organs. It is recognized that the SLC agents of the invention may exert an effect on another aspect of the immune cascade described herein. Thus, by "modulation of dendritic cell function" 5 is intended modulation of receptor binding or dendritic cell activity as well as downstream effects on the immune response and modulation of T cell function. Given its chemotactic activity to both mature DC and T cells, SLC may serve as an important co-localization signal for these cells, mediating the initiation of immune responses. Thus, the compositions of the invention find use in mediating recruitment of leukocytes 10 into sites of inflammation and immune responses, particularly, the chemotaxis of dendritic and other cells. The compositions of the invention furthermore are useful in preventing proliferation of tumor cells, and tumor progression, preventing hyperproliferative cell growth, inhibiting angiogenesis, and generally for the treatment of cancer.

15 By "immunotherapeutic intervention" is intended modulation of an immune response; suppression of an immune response, for example, in preventing graft rejection; prevention and treatment of autoimmune diseases; enhancing an immune response; inhibiting neoplastic or tumor growth; inhibiting angiogenesis; and the like.

By "modulation" of the immune response is intended that the compositions of 20 the invention can be used to promote, enhance, upregulate, or initiate an immune response, or alternatively, can be used to suppress or downregulate an immune response. Importantly, as dendritic cells are involved in initiating the immune response, the compositions of the invention can be used to prevent a primary immune response.

By "primary immune response" is intended the onset or initiation of the immune 25 cascade as described above. Such a response can be prevented at least by blocking the capturing and presenting of antigens as well as the transporting of antigens through the body to stimulate T cells.

The SLC agents of the invention comprise native SLC polypeptides, native SLC nucleic acid sequences, polypeptide and nucleic acid variants, antibodies, monoclonal

antibodies, and other components that are capable of blocking the immune response through manipulation of SLC expression, activity and receptor binding. Such components include, for example, proteins or small molecules that interfere with or enhance SLC promoter activity; proteins or small molecules that attract transcription 5 regulators; polynucleotides, proteins or small molecules that stabilize or degrade SLC mRNA; proteins or small molecules that interfere with receptor binding; and the like.

It is recognized that the invention is not bound by any particular method. Having recognized that SLC is chemotactic to mature dendritic cells, and T cells, any means of suppressing or enhancing SLC activity, for example, by interfering with 10 receptor binding, interfering with SLC promoter activity, interfering with gene expression, mRNA stability, or protein stability, etc., can be used to modulate the primary immune response and are encompassed by the invention. The amino acid and DNA sequence of SLC are known in the art. See, for example, Pachynski *et al.* (1998) *J. Immunol.* 161:952; Yoshida *et al.* (1998) *J. Biol. Chem.* 273:7118; Nagira *et al.* (1998) *Eur. J. Immunol.* 28:1516-1523; Nagira *et al.* (1997) *J. Biol. Chem.* 272:19518. 15 All of which are herein incorporated by reference.

SLC variants, that retain SLC activity, can be utilized to enhance the immune response, prevent tumor progression, prevent hyperproliferative cell growth, and the like. It is recognized that some SLC variants or derivatives may retain the ability to 20 bind with receptor, but will not retain chemotactic activity or the activity essential for initiation of the immune response. Thus, such variants or derivatives will be useful to suppress the immune response. Methods for selecting polypeptides that are capable of suppressing the immune response yet binding chemotactic cells through the SLC receptor are available in the art. Assay systems such as those set forth in the 25 experimental section can be utilized. Combinatorial chemistry approaches can be used to determine SLC variants or derivatives which may be useful to suppress the immune response. Such approaches increase the range of molecular diversity available for selection and greatly broaden the scope of random collections of molecules being surveyed for biological activity. See, generally, John N. Abelson (Ed.) *Methods In*

5 *Enzymology, Combinatorial Chemistry*, 267, Academic Press (1996); U.S. Patent No. 5,270,170; U.S. Patent No. 5,498,530; herein incorporated by reference. Variants of the SLC protein that function as either SLC agonists or antagonists can be identified by screening combinatorial libraries of mutants proteins for SLC protein agonist or antagonist activity.

10 Suitable SLC polypeptides can be SLC variants, SLC fragments, analogues, and derivatives. By "analogues" is intended analogues of either SLC or an SLC fragment that comprise a native SLC sequence and structure having one or more amino acid substitutions, insertions, or deletions. Peptides having one or more peptoids (peptide mimics) are also encompassed by the term analogues (WO 91/04282). By "derivatives" is intended any suitable modification of SLC, SLC fragments, or their respective analogues, such as glycosylation, phosphorylation, or other addition of foreign moieties, so long as the desired activity is retained. Methods for making SLC fragments, analogues, and derivatives are available in the art.

15 The SLC polypeptides or variants thereof of the invention may be utilized to produce antibodies specific for SLC polypeptides and variants thereof. Antibodies may be polyclonal or monoclonal. An intact antibody, or a fragment thereof, such as Fab or F(ab')₂, as well as ScFV can be utilized. Methods for making or selecting antibodies are known in the art. See, for example, Kohler and Milstein (1975) *Nature* 256:495-497 (hybridoma technique); Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp 77-96; Kozbor *et al.* (1983) *Immunol. Today* 4:72; *Current Protocols in Immunology* (1994) Coligan *et al.* (eds.) John Wiley & Sons, Inc., New York, NY; Galfre *et al.* (1977) *Nature* 266:55052; Lerner (1981) *Yale J. Biol. Med.* 54:387-402. Additionally, chimeric and humanized monoclonal antibodies can be 20 produced by recombinant DNA techniques, for example, as described in EPA 171,496; EPA 184,187; WO 86/01533; WO 87/02671; Better *et al.* (1988) *Science* 240:1041; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214; Wood *et al.* (1985) *Nature* 314:446; Morrison (1985) *Science* 229:1202; Di *et al.* (1986) *BioTechniques* 4:214; Beidler *et al.* (1988) *J. Immunol.* 25:1202.

141:4053; Verhoeven *et al.* (1988) *Science* 239:1534; U.S. Patent Nos. 5,225,539 and 4,816,567; Marks *et al.* (1993) *Biotechnology* 11:1145-49; Roberts *et al.* (1993) *Gene* 128:67-69; Figini *et al.* (1998) *Cancer Res.* 58:991-996; Hoogenboom *et al.* (1992) *J. Mol. Biol.* 227:381-88; McGuinness *et al.* (1996) *Nat. Biotechnol.* 14:1149-54; 5 Vaughan *et al.* (1996) *Nat. Biotechnol.* 14:309-314; Stevens *et al.* (1998) *J. Biol. Chem.* 273:2874-84; Winter *et al.* (1994) *Annu. Rev. Immunol.* 12:433-455; Winter *et al.* (1993) *Immunol. Today* 14:243-6; McCafferty *et al.* (1990) *Nature* 348:552-4; Gorman (1990) *Semin. Immunol.* 2:457-66.

The antibodies can be applied in a therapeutic context in which treatment 10 involves inhibiting SLC function. An antibody can be used, for example, to block SLC binding. Antibodies can be prepared against specific fragments containing sites required for function e.g. binding.

Anti-antibodies, such as anti-idiotypic antibodies, can also be prepared wherein 15 such anti-antibodies are capable of binding receptor and preventing SLC activation of the immune response. Such antibodies are also useful in a therapeutic context in which inhibition of SLC activity is desired.

As stated above, SLC agents of the invention also comprise polynucleotides encoding the SLC polypeptide, derivatives, analogues, variants, or fragments thereof. The compositions further comprise variant polynucleotides and fragments thereof. such 20 "variant polynucleotides" include those that encode the SLC polypeptide but differ from the native sequence due to the degeneracy of the genetic code. Variant nucleotide sequences also include synthetically derived nucleotide sequences that have been generated, for example, by using site-directed mutagenesis. Generally nucleotide sequence variants of the invention will have at least about 70%, preferably at least about 25 80%, more preferably about 90 to 95% or more, and most preferably about 98% or more sequence identity to SEQ ID NO:1 or 3.

Polynucleotides of the invention may be naturally occurring, such as allelic variants, homologs, orthologs, or may be constructed by recombinant DNA methods or by chemical synthesis. Alternatively, the variant polypeptides may be non-naturally

occurring and made by techniques known in the art, including mutagenesis.

Polynucleotide variants may contain nucleotide substitutions, deletions, inversions and insertions.

Polynucleotide sequences of the invention include antisense nucleotides.

5 Antisense technology utilizes nucleic acid base-pair recognition and achieves a high degree of specificity in modulating gene expression. Generally, small, synthetic oligonucleotides resembling single-stranded DNA are used intracellularly to hybridize the coding sequences in a specific messenger RNA (mRNA) target by Watson-Crick base pairing. Antisense oligodeoxynucleotides have been recognized as a major class of

10 potential pharmaceuticals. Antisense oligodeoxynucleotides (ODNs) used in the invention are capable of complementary binding to the SLC mRNA and modulating SLC activity.

The sequences of the ODNs used as antisense molecules can be substantially complementary to any portion of the SLC mRNA sequences or SLC promoter regions.

15 Preferably, the antisense sequences are not identical or complementary to a sequence for another mRNA or to a rRNA or tRNA. Generally, ODNs are about 40% to about 100% complementary, preferably about 50% to about 85%, more preferably about 60% to about 80% complementary to a portion of the selected SLC sequence. In one embodiment of the invention, the ODNs sequence can be designed to be significantly complementary, that is about 80% to about 100% complementary to the SLC mRNA sequence adjacent to the translation initiation codon AUG.

The length of the ODNs can range from about 10 nucleotides to about 300 nucleotides, preferably from about 10 nucleotides to about 100 nucleotides, and more preferably from about 15 nucleotides to about 50 nucleotides.

25 Methods are generally available in the art for expression of the polynucleotides of the invention. See, for example, Sambrook *et al.*, Cold Spring Harbor, NY, (1989). A variety of expression vectors can be used to express the polynucleotides of the invention. Vectors containing the appropriate polynucleotide can be introduced into host cells for propagation and expression using well-known techniques. See, for

example, Studier *et al.*, *Gene Expression Technology: Methods In Enzymology* 185:60-89 (1990); Wada *et al.* (1992) *Nucleic Acids Res.* 20:2111-2118; Schultz *et al.* (1987) *Gene* 54:113-123; Lucklow *et al.* (1989) *Virology* 170:31-39; Seed, B. (1987) *Nature* 329:840; Kaufman *et al.* (1987) *EMBO J.* 6:187-195; and Smith *et al.* (1988) *Gene* 67:31.

The protein and small molecule agents discussed above can be identified by methods available in the art. For example, as discussed above, using combinatorial chemistry or phage display approaches peptides can be generated at random and selected for receptor binding or the ability to modulate SLC activity.

10 The SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, SLC polynucleotides encoding said polypeptides, variants and fragments, and the SLC agents useful in the methods of the invention can be incorporated into pharmaceutical compositions suitable for administration into a subject. Subjects include mammals, e.g. humans, dogs, cattle, horses, etc. Such compositions typically comprise at least one
15 SLC polypeptide, SLC polypeptide variant, SLC polypeptide fragment, SLC polynucleotide encoding said polypeptide, variant or fragment, an SLC agent, or a combination thereof, and a pharmaceutically acceptable carrier. Methods for formulating the SLC compounds of the invention for pharmaceutical administration are known to those of skill in the art. See, for example, Remington: The Science and
20 Practice of Pharmacy, 19th Edition, Gennaro (ed.) 1995, Mack Publishing Company, Easton, PA.

As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated

into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration.

The route of administration will vary depending on the desired outcome. Generally for initiation of an immune response, injection of the agent at or near the 5 desired site of inflammation or response is utilized. Alternatively other routes of administration may be warranted depending upon the disease condition. That is, for suppression of neoplastic or tumor growth, injection of the pharmaceutical composition at or near the tumor site is preferred. Alternatively, for prevention of graft rejection, systemic administration may be used. Likewise, for the treatment or prevention of 10 autoimmune diseases systemic administration may be preferred. Examples of routes of systemic administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline 15 solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or 20 sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, 25 suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a

solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a protein or antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the

composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc.

20 Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are

dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used 5 as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. 5,328,470), implantation or by stereotactic injection (see e.g., Chen *et al.*, *PNAS* 91:3054-3057 (1994)). Vectors for expression in mammalian hosts are disclosed in Wu *et al.* (1991) *J. Biol. Chem.* 266:14338; Wu and Wu (1988) *J. Biol. Chem.* 263:14621; and Zenke *et al.* (1990) *Proc. 10 Nat'l. Acad. Sci. USA* 87:3655; all of which are herein incorporated by reference. The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical 15 preparation can include one or more cells which produce the gene delivery system.

In one embodiment, the pharmaceutical composition can be delivered via slow release formulation or matrix comprising SLC protein or DNA constructs suitable for expression of SLC protein into or around a site within the body. In this manner, a transient lymph node can be created at a desired implant location to attract dendritic 20 cells and T cells initiating an immune response.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. That result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease or any other desired alteration of a biological system.

25 The pharmaceutical compositions of the invention, comprising SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding said SLC polypeptides, variants and fragments, as well as SLC agents, as defined above, are administered in therapeutically effective amounts. The "therapeutically effective amount" refers to a nontoxic dosage level sufficient to induce

a desired biological result. Amounts for administration may vary based upon the desired activity, the diseased state of the mammal being treated, the dosage form, method of administration, patient factors such as age, sex, and severity of disease. It is recognized that a therapeutically effective amount is provided in a broad range of 5 concentrations. Such range can be determined based on binding assays, chemotaxis assays, and *in vivo* assays. Generally, pharmaceutical compositions will comprise from about 0.1 ng/ml to about 1 mg/ml; from about 1 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml to about 600 ng/ml, 700 ng/ml, 800 ng/ml, 900 ng/ml, 1 μ g/ml, 10 μ g/ml, 100 μ g/ml, 200 μ g/ml, 300 μ g/ml, 400 μ g/ml, 500 μ 10 g/ml, 900 μ g/ml, 1 mg/ml. These compositions can be used for injection at a particular target site (injection of about 20 μ l, 500 μ l from 30 μ l, 40 μ l, 50 μ l, 75 μ l, 100 μ l, to about 200 μ l, 300 μ l, 400 μ l, 500 μ l, 1 ml, 2 ml, 3 ml, 4 ml, 5 ml and up to about 10 ml), or alternatively for other administration.

15 Regimens of administration may vary. A single injection or multiple injections of the agent may be used. Likewise, expression vectors can be used at a target site for continuous expression of the agent. Such regimens will vary depending on the severity of the disease and the desired outcome.

20 In a preferred embodiment, an SLC or SLC composition is injected directly into the tumor or into a peritumor site. By peritumor site is meant a site less than about 15 cm from an outer edge of the tumor. SLC administration may be to one or more sites. Preferably, SLC administration is at multiple sites within a tumor and/or surrounding a tumor.

25 In one embodiment, the cancer or hyperproliferative cell growth is surgically removed before and/or after administration of the SLC. Thus, in one embodiment, the invention comprises a method of treating a cancer, tumor, or hyperproliferative disorder, comprising: (a) administering said SLC to the site of cancer, tumor, or hyperproliferative cell growth; (b) surgically removing said cancer or hyperproliferative cell growth; and (c) administering said SLC to the site of surgical removal.

The SLC compositions of the invention may be co-administered or co-formulated with other agents that may participate in the inhibition of cancer, angiogenesis, hyperproliferative cell growth or the modulation of an immune response. Examples of such agents include methotrexate, tamoxifen, nelandron, nilutamide, 5 adriamycin, 5FU, interferons and the like. A preferred agent is IL-2. Preferably, the IL-2 is recombinant human IL-2. Most preferably the recombinant IL-2 is Proleukin™ (Chiron Corp., Emeryville, CA). Preferred routes of administration of IL-2 include, but are not limited to, IV, SQ, oral, mucosal, intranasal, dermal, i.p., intra-tumor and peritumor routes of administration. Most preferably, IL-2 is administered by IV or SQ 10 to humans.

Proleukin may be obtained in a lyophilized powder for IV(SQ) injection containing 22 million IU/Vial. To prepare for injection, 1.2 ml sterile water is added to the vial to make 18 million IU/ml (or 1.3 mg protein /ml). This solution is stable at room temperature for 48 hours. Albumin must be added to D5W before addition of 15 Proleukin. Add 1 ml of 5% albumin (or 0.2ml of 25% albumin) to each 50ml of D5W to make D5W with 0.1% albumin. Final dose can be diluted to 5-60 mcg Protein/ml in D5W with 0.1% Albumin (see above)

Dosage widely varies from high dose protocols of 600,000 IU/kg to low dose protocols giving 6 million IU/M2. Routes vary from SQ to IV - continuous or bolus 20 (Given by IV push over 30 minutes or less).

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL
Materials and Methods

Chemokines and antibodies

5 Human recombinant SLC (MW = 12 kD), ELC (MW = 8.8 kD) and RANTES (MW = 7.8 kD), as well as phycoerythrin (PE)-conjugated anti-human CCR6 antibodies were purchased from R&D Systems (Minneapolis, MN). PE-conjugated anti-human CD1a, CD14 and fluorescein isothiocyanate (FITC)-conjugated anti-human CD86, CD40 and HLA-DR antibodies were obtained from Pharmingen (San Diego, CA). PE-conjugated anti-human CD83 was obtained from Immunotech/Coulter (Miami, FL).

10

Generation of DC

DC were derived from peripheral blood monocytes as previously described (Bender *et al.* (1996) *J. Immunol. Methods* 196:121). Buffy coats from healthy donors 15 were obtained from the Blood Center of the Pacific (San Francisco, CA). CD14⁺ monocytes were isolated by negative depletion using Monocyte Isolation Kits along with MACS columns (Miltenyi Biotec, Auburn, CA). Cells were cultured at 0.6×10^6 per ml in medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Summit, Inc., Fort Collins, CO), 2 mM glutamine, penicillin and streptomycin 20 containing 1,000 U/ml recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (Peprotech, Rocky Hill, CA) and 1,000 U/ml rhIL-4 (Peprotech). Fresh culture medium with cytokines was added every two days. Monocyte-conditioned medium (MCM) was prepared as previously described (Bender *et al.* (1996) *J. Immunol. Methods* 196:121) and was added to DC cultures at 30% on either 25 the 5th or 6th day following culture initiation for 3 additional days. In some experiments, cells were matured using 50 ng/ml recombinant human TNF α (Cetus, Emeryville, CA) instead of MCM. Expression of surface markers upon MCM-treatment was analyzed by flow cytometry.

Chemotaxis assays

Migration assays were performed as described previously (Rubbert *et al.* (1998) *J. Immunol.* 160:3933) with slight modifications. Briefly, 10,000 to 15,000 cells were added to each of the top chamber of 96 well microchemotaxis plates (101-5 5 Neuroprobe, Cabin John, MD). Microchemotaxis plates were incubated at 37°C for 2 hours. The number of cells in the bottom chamber was measured using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Indianapolis, IN) according to manufacturer's protocol. Each measurement was set up in triplicate and the average values and standard deviation were calculated. In some measurements the cells were 10 preincubated with 100 ng/ml of pertussis toxin (PT) for 2 hr at 37°C.

Calcium mobilization

Cells were incubated at 10⁶ per ml of medium (see above) plus 10 mM HEPES with 2 µM Fluo-3AM (Molecular Probes, Eugene, OR) at 37°C for 30 minutes. Cells 15 were washed and subsequently incubated with 1 µg/ml propidium iodide for 15 minutes at room temperature. Cells were washed again and were resuspended at 4 x 10⁵ per ml in medium with 10 mM HEPES. Fluo-3 fluorescence of viable cells (based on propidium iodide exclusion) was analyzed by flow cytometry.

20 Flow cytometric analysis

Analysis was performed on a FACScan (Becton Dickinson, Franklin Lakes, NJ) and the data acquired to a Macintosh 7100 (Apple, Cupertino, CA) running CellQuest v3.1 software. The acquired data was analyzed and displayed using FlowJo (Tree Star Inc, San Carlos, CA).

25

Quantification of RNA expression by RT-PCR and Light Cycler

RT-PCR: Total RNA was isolated from cells using Trizol reagent (Gibco/BRL) according to the manufacturer's protocol. After treatment with RNase-free DNase, first strand cDNA was synthesized by priming with oligo-dT₁₂₋₁₈ using SuperScriptII

Preamplification System (Gibco/BRL, Gaithersburg, MD) following manufacturer's protocol. After RNaseH treatment, the cDNA was quantitated by spectrophotometric methods. Based on the published human sequences for CCR1-9, CXCR1-5, CX₃CR1 and three orphan receptors, PCR oligonucleotides targeted to the regions close to the 3' end of the coding regions with the highest divergence among chemokine receptors were designed. The forward (f) and reverse (r) primers, the SEQ ID NO., the expected fragment size and the GenBank accession numbers for each gene is indicated as follows.

CCR1 (f: TGACTCTGGGGATGCAAC,
r: TCCACTCTCGTAGGCTTCG, SEQ ID NO:7, 538 bp, L10918).

10 CCR2 (f: CCCTTATTTCCACGAGGATGG, SEQ ID NO:8,
r: CGCTTGGTGTGCTTCG, SEQ ID NO:9, 407 bp, U03905).

CCR3 (f: TGAGACTGAAGAGAGTTGTTG, SEQ ID NO:10,
r: ATTGATAGGAAGAGAGAAGG, SEQ ID NO:11, 280 bp, U51241).

CCR4 (f: CAGCTCCCTGGAAATCAACATTC, SEQ ID NO:12,
r: CAGTCTTGGCAGAGCACAAAAAGG, SEQ ID NO:13, 369 bp, X85740).

15 CCR5 (f: CCAAAAGCACATTGCCAAACG, SEQ ID NO:14,
r: ACTTGAGTCCGTGTCACAAGCC, SEQ ID NO:15, 136 bp, X91492).

CCR6 (f: ATCCTGCCAGAGCGAAAAGC, SEQ ID NO:16,
r: CATTGTCGTTATCTGCGGTCTCAC, SEQ ID NO:17, 248 bp, U68032).

20 CCR7 (f: TGCCATCTACAAGATGAGCT, SEQ ID NO:18,
r: GGTGCTACTGGTGTGTTGA, SEQ ID NO:19, 492 bp, L08176).

CCR8 (f: TCTGAAGATGGTGTCTACA, SEQ ID NO:20,
r: ACTTTCACAGCTCTCCCTA, SEQ ID NO:21, 486 bp, U45983).

CCR9 (f: GCATGGGACCATTGGAAGC, SEQ ID NO:22,
r: CAGTCATTCCCTTGGCAGTAAG, SEQ ID NO:23, 478 bp, Y12815).

25 CXCR1 (f: CCTTCTTCCTTCCGCCAG, SEQ ID NO:24,
r: AAGTGTAGGAGGTAACACGATGACG, SEQ ID NO:25, 512 bp, L19591).

CXCR2 (f: CTTTCCGAAGGACCGTCTACTC, SEQ ID NO:26,
r: TGTGCCCTGAAGAAGAGCCAAC, SEQ ID NO:27, 545 bp, M73969).

CXCR3 (f: AATACAACCTCCCACAGGTG, SEQ ID NO:28,
r: CAAGAGCAGCATCCACATCC, SEQ ID NO:29, 391 bp, X95876).
CXCR4 (f: GCTGTTGGCTGAAAAGGTGGTC, SEQ ID NO:30,
r: CACCTCGCTTCCTTGGAGA, SEQ ID NO:31, 538 bp, X71635).
5 CXCR5 (f: ACGTTGCACCTCTCCCAAGAG, SEQ ID NO:32,
r: AGAGAGCCATTCACTAGCTGCAGG, SEQ ID NO:33, 299 bp, X68149).
Bonzo (f: TTACCATGACGAGGCAATTCC, SEQ ID NO:34,
r: ATAACTGGAACATGCTGGTGGC, SEQ ID NO:35, 484 bp, af007545).
V28 (f: TGAATGCCTTGGTGAATCCCC, SEQ ID NO:36,
10 r: GGAGAAATCAACGTGGACTGAGC, SEQ ID NO:37, 456 bp, U20350).
GPR5 (f: CTCCTCAATATGATCTTCTCCAT, SEQ ID NO:38,
r: TCTGCAGAACAGGGTGAA, SEQ ID NO:39, 438 bp, L36149).
GPR-9-6 (f: GCCATGAGAGCACATACATTG, SEQ ID NO:40,
r: GCAGATGTCAATGTTGGTGGA, SEQ ID NO:41, 441 bp, U45982).
15 GAPDH primers were from Clontech (Palo Alto, CA, #5406). For each PCR reaction, 0.5 µg of cDNA was used in 25 µl reactions containing 10 mM Tris, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3, 1.25 U Taq DNA polymerase (Boehringer Mannheim), 0.2 mM dNTP and 50 pmol of each primer. PCR reactions were carried out at 94°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute for 30 cycles and analyzed on 2% agarose gels.
20 Some PCR reactions were carried out with a 1-minute annealing step at 55°C instead of 60°C. The specificity of each pair of primers for their respective gene was confirmed by cloning each of the PCR products into pCR2.1-TOPO (Invitrogen; Carlsbad, CA) and sequence verified (data not shown).

25 *Light Cycler* (Idaho Technology, Idaho Falls, ID): Optimal light cycling conditions were used for these semi-quantitative PCR reactions. Each light cycling reaction (10 µl) contained: 50mM Tris-HCl pH 8.3, 2.5mM MgCl₂, 250µg/ml bovine serum albumin (BSA), 0.2mM dNTP's, 1U DNA Taq Polymerase, 0.1 pmol of each primer, and 1:5000 SYBR Green I (Molecular Probes). Four reactions were run for each primer set to establish a concentration curve (250 ng, 125 ng, 62.5 ng and 31.25 ng

cDNA) for dye intercalation calculations. The Light Cycler program consists of one normalization cycle (65°C to 85°C to 95°C, hold 20 seconds), followed by 45 cycles of: denaturation (95°C for 5 seconds), annealing (3°C below T_m for 2 seconds) and extension (72°C for 30 seconds). The 45 cycles are followed by a single “Big Melt” step (60°C for 5 seconds, 95°C for 5 seconds and 32°C for 20 seconds). Each pair of primers have 2°C or less difference in T_m and generate a product within the optimal range (100-500 bp) for SYBR Green I dye quantification. The sequences and annealing temperatures of the primers used are: CCR1 (58°C), CCR2 (62°C), CCR3 (62°C), CCR4 (60°C), CCR5 (58°C), CCR6 (same as RT-PCR, 60°C), CCR7 (58°C) and actin (57°C). Because actin amplifications gave similar profiles for both the immature and mature DC cDNA samples, actin was used as a reference and all CCR samples were evaluated as to their abundance with respect to the actin of that cDNA sample.

Example 1

15 Generation of Immature and Mature Dendritic Cells

Immature DC were derived from monocytes isolated from human peripheral blood using GM-CSF and IL-4. To generate mature DC, we treated the cells with monocyte-conditioned media (MCM) or TNF α . MCM-treatment induced significant upregulation of markers associated with DC maturation such as CD86, CD83 and MHC class II surface expression. The mean fluorescence channels for DC without or with MCM-treatment were 417 and 3624 for CD86, 186 and 721 for CD83, 667 and 790 for CD40, 202 and 132 for CD14, 2401 and 2413 for CD1a and 1242 and 3106 for HLA-DR respectively. These cells were also tested in an allogeneic MLR assay and the average stimulation indices at a DC:T ratio of 1:100 were 17.6 and 47.2 for DC before and after MCM-treatment respectively (data not shown). Therefore, the DC differentiated in this *in vitro* culture model resemble their *in vivo* counterparts by both immunophenotype and function.

Example 2
SLC and ELC are Chemotactic to Mature DC

5 ELC, a chemokine expressed in secondary lymphoid organs (Dieu *et al.* (1998) *J. Exp. Med.* 188:373; Yoshida *et al.* (1997) *J. Biol. Chem.* 272:13803; Rossi *et al.* (1997) *J. Immunol.* 158:1033) has previously been demonstrated to be chemotactic for mature DC. SLC is another chemokine expressed in lymphoid tissues previously shown to be chemotactic for naïve T cells. CCR7 is a receptor for SLC and ELC and is
10 upregulated upon DC maturity (Gunn *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:258; Willimann *et al.* (1998) *Eur. J. Immunol.* 28:2025; Yoshida *et al.* (1998) *J. Biol. Chem.* 273:7118; Sozzani *et al.* (1998) *J. Immunol.* 161:1083; Dieu *et al.* (1998) *J. Exp. Med.* 188:373; Sallusto *et al.* (1998) *Eur. J. Immunol.* 28:2760). The experiments tested whether SLC was also chemotactic to mature DC using a Boyden chamber assay. A
15 dose dependent increase in the migration of MCM-treated (mature) DC in response to both SLC (Figure 1a) and ELC (Figure 1b) was observed. In contrast, mature DC did not respond to RANTES (Figure 1c), a chemokine previously demonstrated to be chemotactic to immature DC (Sozzani *et al.* (1995) *J. Immunol.* 155, 7:3292). Similar results were also obtained with DC that were treated with TNF \square instead of MCM
20 (Figure 1a-b). The migratory response of DC to SLC and ELC was dependent on the DC maturation stage since immature DC did not respond to either SLC or ELC (Figures 1a,b) but were able to migrate in response to RANTES (Figure 1c).

25 The migratory responses to SLC and ELC by mature DC were chemotactic and not due to chemokinesis since migration was not observed in the absence of a chemokine gradient (rightmost column, Figure 1a, b). Pretreatment of mature DC with pertussis toxin completely inhibited their migration towards both SLC and ELC (Figure 1d), suggesting that chemotaxis of mature DC by these two chemokines was mediated by G $_{\alpha i}$ coupled- receptor(s) (Baggiolini *et al.* (1997) *Annu. Rev. Immunol.* 15:675).

Example 3

Mobilization of intracellular calcium by mature DC in response to SLC and ELC

Since chemokines can cause rapid increases in intracellular calcium in 5 responding cells (Baggiolini *et al.* (1997) *Annu. Rev. Immunol.* 15:675), we next tested whether SLC and ELC induced calcium mobilization in our mature and immature DC. SLC and ELC both induced rapid calcium mobilization in mature DC (Figure 2b,c), but not in immature DC (Figure 2a). In contrast, RANTES triggered a rapid calcium response in immature DC (Figure 2a), but not in mature DC (Figure 2b,c). These 10 results closely paralleled the differential chemotactic responses observed in migration assays (Figure 1). As with the chemotaxis response, the calcium responses of MCM-treated cells to SLC and ELC were dose dependent (Figure 2d-e).

Example 4

15 Cross-desensitization of SLC and ELC Induced Ca^{2+} Mobilization

It was consistently observed that SLC induced a stronger calcium response in mature DC than ELC at equivalent doses (Figure 2). Also, SLC-induced migration of mature DC has an ED_{50} of 6 ng/ml while that for ELC was 35 ng/ml (Figure 1a,b). 20 Taken together, these observations suggested that SLC from our commercial source has a higher specific activity than ELC. Indeed, pretreatment of DC with 100 ng/ml of SLC was sufficient to completely inhibit the calcium response to 500 ng/ml of ELC (Figure 2f). Conversely, pretreatment with 500 ng/ml of ELC abrogated most of the SLC-induced calcium response (Figure 2g). It has previously been demonstrated in CCR7-transfected cells that SLC and ELC can cross-desensitize each other in calcium 25 mobilization (Willimann *et al.* (1998) *Eur. J. Immunol.* 28:2025; Yoshida *et al.* (1998) *J. Biol. Chem.* 273:7118). Our results indicated that SLC and ELC also share similar signaling pathways in DC. It was recently reported that mature DC produce ELC in an autocrine fashion. Therefore, we also examined the mRNA expression of SLC in our

immature and mature DC by RT-PCR but did not find either cell population to express SLC mRNA (data not shown).

Example 5

5 Chemokine Receptor mRNA Expression Before and After MCM Treatment

Given the usefulness of chemokine receptor upregulation as an indicator, a comprehensive survey of chemokine receptor expression on DC before and after MCM-treatment was carried out. Significant increases in mRNA expression of CCR4, CCR7 and CXCR5/BLR1, as well as decreases in CCR1, CCR5 and CXCR2 were observed upon DC maturation (Figure 3). CXCR1 expression was also consistently reduced upon MCM-treatment, despite a low basal level. A slight decrease in CCR2 and a slight increase in CCR8 were also observed, but no significant changes for CCR3, CCR6, CCR9, CXCR3 and CXCR4 were detected. No message for the orphan receptor GPR-9-15 6 was detected and there were no differences in the expression levels of CX₃CR1, Bonzo and GPR5 in mature and immature DC (data not shown). To confirm the validity of the semi-quantitative RT-PCR results, the expression levels of receptors reportedly involved in the binding of RANTES, ELC and SLC (CCR1, 3-7) were examined using SYBR green/Light Cycler. As shown in Table I, the quantitative data 20 from the Light Cycler closely parallel the trends observed with the results from agarose gel analysis (Figure 3). Sallusto and coworkers recently reported similar changes in the expression of CCR1, CCR4, CCR5, CCR7 and CXCR1 using an RNase-protection assay (Sallusto *et al.* (1998) *Eur. J. Immunol.* 28:2760).

CCR6 RNA was previously reported to be undetectable in monocyte-derived 25 DC (Power *et al.* (1997) *J. Exp. Med.* 186:825; Greaves *et al.* (1997) *J. Exp. Med.* 186:837). In contrast, we were able to detect CCR6 mRNA expression in our monocyte-derived DC but did not observe significant changes in expression upon MCM-treatment (Figure 3). We also obtained similar results when using the primers used by Power *et al.* (1997) *J. Exp. Med.* 186:825 (data not shown). The identity of our 30 PCR product was also confirmed by direct sequencing indicating that CCR6 was indeed

expressed at the mRNA level (data not shown). However, flow cytometric analysis of both mature and immature DC using an antibody to human CCR6 indicated that there was no surface expression of CCR6 on either cell type (data not shown).

The observation that CCR4 and CXCR5 are upregulated upon MCM
5 treatment of DC is intriguing since these two receptors have been demonstrated to bind MDC (Godiska *et al.* (1997) *J. Exp. Med.* 185:1595; Imai *et al.* (1998) *J. Biol. Chem.* 273:1764) and BLC/BCA-1 (Legler *et al.* (1998) *J. Exp. Med.* 187:655; Gunn *et al.* (1998) *Nature* 391:799) respectively. MDC is expressed in the thymus and at a lower level in the spleen, and has been shown to be chemotactic for monocyte-derived DC
10 (Godiska *et al.* (1997) *J. Exp. Med.* 185:1595). The mouse homologue of MDC was also found to be expressed in the T cell area of the lymph node adjacent to the B cell follicles (Tang *et al.* (1998) *J. Leukoc. Biol.* 64:81). On the other hand, BLC is expressed in the follicles of Peyer's patches, the spleen and lymph nodes (Gunn *et al.* (1998) *Nature* 391:799). Thus, the upregulation of CCR4 and CXCR5 may allow DC
15 to sense additional chemokines guiding them for entry into secondary lymphoid organs. Once in secondary lymphoid organs, DC attracts naive T cells by secreting chemokines such as DC-CK1, initiating immune responses. Randolph *et al.* (Science (1998) 282:480) reported that the differentiation of monocytes into DC upon traversing endothelial monolayers, a culture system that mimics the *in vivo* movement of DC from
20 tissues into lymphatic vessels.

In summary, we have demonstrated that SLC, like ELC, is a chemoattractant for mature but not immature DC. The upregulation of CCR7 on DC upon MCM-treatment is consistent with this finding. It is likely that the downregulation of receptors for inflammatory chemokines (CCR1, CCR5, CXCR2) and the upregulation of receptors on
25 mature DC for chemokines expressed in secondary lymphoid organs provide a mechanism by which DC leave the site of inflammation and antigen uptake to migrate to regional lymphoid organs in order to initiate immune responses.

Table 1. Light Cycler quantification of chemokine receptor expression in cDNA from immature and mature DC.

Receptor	FOLD CHANGE AFTER MCM
5 CCR1	-4.3
CCR3	-2.1
CCR4	+3.3
CCR5	-3.1
CCR6	-1.5
10 CCR7	+24.0

15 cDNA samples from immature (-MCM) or mature (+MCM) DC were used in light cycle PCR reactions as described in Materials and Methods. The amount of product for each PCR reaction is calculated from the incorporation of fluorescent dye, SYBR green, and represented as a percentage of the amount of actin product obtained from the same cDNA sample. Results are expressed as the fold change upon addition of MCM. These data are representative of duplicates for one cDNA sample and repeats upon three independent sets of cDNA.

20 Example 6
Inhibition of Tumor Growth in Mice Using SLC

The mouse melanoma cell line, B16-BL6, was used to establish subcutaneous tumors in 6-8 week old pink-skinned female BDF-1 mice (Charles River Laboratories, Boston, Massachusetts). B16-BL6 cells were maintained as monolayers in EMEM media (Bio-Whittaker, Walkersville, MD) containing 10% fetal bovine serum and 20 mM L-glutamine (Sigma, St. Louis, MO). Cells were harvested by treatment with trypsin for 1-2 minutes and then washed with media. The cells were resuspended in media at a concentration of 5×10^6 cells/ml for subsequent injection into mice.

25 To produce subcutaneous tumors, 10^6 B16-BL6 cells in 0.2 ml media were injected into upper back region of 6-8 weeks old female BDF-1 mice with 27.5 gauge needles on day 0. Cell viability was assessed by the trypan-blue exclusion dye method before and after cell injection. The number of dead cells before injection was not more

than 10% of total cells. By day 6, tumor diameter was approximately 5-10 mm. Tumor volume was then measured daily from days 6 to 27.

The effect of SLC on tumor growth was assessed. Recombinant mouse SLC was produced using a baculovirus expression system and was purified on S1 and heparin columns. Purified mouse SLC was diluted into PBS, pH 7.4 prior to injection. All injections of SLC were administered subcutaneously at days 3 and 4 in two 0.02 ml injections per day, for a total injection volume of 0.04 ml per day. The SLC injection was a different site each time, and each site was approximately 3 mm away from the edge of tumor. The concentrations of SLC injected ranged from 0.025-250 μ g/ml in PBS. PBS was used as a negative control, and 10 mg/ml. Dacarbazine (DTIC; Sigma, catalog # D2390) was used as a positive control for inhibition of tumor growth. Some mice were sacrificed at two or four days after SLC injection. All remaining mice were sacrificed after tumor volume reached 4000 mm³. Tissue specimens were collected immediately after sacrificing, embedded in OCT gel (Sakura Finetek) and frozen in the dry ice pre-cold 2-Methylbutane.

The results of an experiment testing the effect of SLC treatment on BDF-1 mouse survival are shown in Figure 4. As shown in Figure 4, treatment with SLC inhibited tumor growth and was most effective at a concentrations of 0.25 and 25 μ g/ml (1 and 100 ng/day). The results from a series of similar experiments show that SLC or DTIC treatment of BDF-1 mice having B16-BL6 tumors increases survival (tumors < 2000 mm³) from 18 days in the control group to 27 days in the SLC and DTIC treatment groups.

The rates of tumor growth in SLC or DTIC treated and control BDF-1 mice are shown in Figure 5. Maximum primary tumor growth inhibition occurred on day 13. SLC (25 μ g/ml) inhibited tumor growth by 58%. DTIC inhibited tumor growth by 69% (Figure 6).

The tumor and surrounding tissue were analyzed for the presence of immune cells by immunofluorescence staining. Naïve T cells were detected at two days after SLC injection with nodule formation at 100 μ g/ml SLC. In contrast, naïve T cells were

not detected two days after SLC injection in the PBS control, nor in mice treated with 250 μ g/ml SLC. Four days after SLC treatment, activated T cells with nodule formation were detected in mice treated with 100 μ g/ml SLC, but not in mice treated with PBS or 250 μ g/ml SLC. Eleven days after treatment with SLC, activated T cell 5 tumor infiltration was detected in tumors treated with 10 and 100 μ g/ml SLC, but not in tumors treated with PBS or 250 μ g/ml SLC.

At two and four days after SLC injection, mature dendritic cells were detected in a dose dependent manner with nodule formation at SLC concentrations of 10 μ g/ml and 100 μ g/ml. Dendritic cells were not detected in the negative PBS controls or in tumor 10 sites treated with 250 μ g/ml SLC. Macrophages were detected in both SLC and PBS treated tumors. In contrast, B cells, NK cell neutrophils, basophils and eosinophils were not detected in SLC nor PBS treated tumor sites.

The ability of SLC to inhibit tumor formation in mice injected subcutaneously with CT26-13 colon cancer cells was tested in a series of experiments similar to those 15 described above. Maximum inhibition of tumor growth occurred at day 25. Administration of 25 or 75 μ g/ml SLC at days 11 and 12 inhibited CT26-13 tumor growth by 43% at day 25, while 250 μ g/ml SLC inhibited tumor growth by 19%.

Example 7

Inhibition of Tumor Growth in Mice Using SLC and IL-2 Combination Therapy 20

The ability of SLC in combination with IL-2 to inhibit the growth of B16-BL6 melanoma cells implanted subcutaneously into BDF-1 mice was tested. B16-BL6 mouse melanoma cells were prepared and injected subcutaneously in to BDF-1 mice on day 0 as described in Example 6. SLC (0.25 μ g/ml or 2.5 μ g/ml in two 0.02 ml 25 injections per day) was administered on days 3 and 4 as described above. Treatment with PBS, pH 7.4, on days 3 and 4 was performed as the negative control. Treatment with 0.2 ml 0.01% DTIC in 0.05N HCl, administered by i.p. injection on days 3-9 was performed as the positive control.

IL-2 was prepared for injection as follows. 16.8 ml 1.1 mg/ml IL-2 30 (ProleukinTM, Chiron corporation, Emeryville, CA) was added to 0.74 ml 5% human

serum albumin (HAS) and 19.44 ml 5% dextrose. IL-2 was administered daily by IV injection in 0.2 ml on days 3-9.

The results are shown in Figure 9. Treatment with the positive control, DTIC, reduced tumor growth by approximately 70% at day 14. Treatment with 2.5 μ g/ml SLC 5 alone, IL-2 alone or 2.5 μ g/ml SLC in combination with IL-2 reduced tumor growth by approximately 60% at day 14. Treatment with 0.25 μ g/ml SLC in combination with IL-2 had the greatest effect, reducing tumor growth by approximately 85%.

All publications and patent applications mentioned in the specification are 10 indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

15 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method of treating cancer or hyperproliferative disorder in a mammalian subject, comprising: administering a therapeutically effective amount of an SLC to said subject.
5
2. The method of claim 1, wherein said SLC is a human SLC.
3. The method of claim 2, wherein said human SLC has the polypeptide sequence of SEQ ID NO:5.
10
4. The method of claim 3, wherein said human SLC has at least 70% sequence identity with the polypeptide of SEQ ID NO:5.
5. The method of claim 1, wherein said SLC is an active variant of the polypeptide of SEQ ID NO:5 or 6;
15
wherein said variant has at least 30% of the angiostatic activity, anti-tumor activity and/or the dendritic cell-chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and
20
said derivative has at least 70% sequence identity with the polypeptide of SEQ ID NO:5 or 6;
6. The method of claim 1, wherein said SLC is an active fragment of the polypeptide of SEQ ID NO:5 or 6;
25
wherein said fragment has at least 30% of the angiostatic activity, anti-tumor activity, and/or the dendritic cell chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and
said fragment comprises at least 10 contiguous amino acid residues of SEQ ID NO:5 or 6.

7. The method of claim 1, wherein said cancer is a solid tumor.
8. The method of claim 7, wherein said tumor is a melanoma.
9. The method of claim 7, wherein said administration is by injection into said tumor.
10. The method of claim 7, wherein said administration is by peritumor injection into one or more sites within 15 cm from an edge of said tumor.
11. The method of claim 1, wherein the administration of said SLC comprises: introducing an SLC polynucleotide into said mammal.
12. The method of claim 11, wherein said SLC polynucleotide is selected from the group consisting of:
 - a) the polynucleotide of SEQ ID NO:1 or 3;
 - b) a polynucleotide encoding the polypeptide of SEQ ID NO:5 or 6;
 - c) a polynucleotide encoding a polypeptide having at least 70% sequence identity to SEQ ID NO:5 or 6, wherein said polypeptide has at least 30% of the angiostatic activity, tumor-inhibiting activity, and/or the dendritic cell-chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and
 - d) a polynucleotide encoding a fragment of the polypeptide of SEQ ID NO:5 or 6, wherein said fragment has at least 30% of the angiostatic activity, tumor-inhibiting activity, and/or the dendritic cell-chemoattractant activity of the polypeptide of SEQ ID NO:5 or 6; and said fragment comprises at least 10 contiguous amino acid residues of SEQ ID NO:5 or 6.

13. The method of claim 1, further comprising the administration of a therapeutically effective amount of an IL-2 in combination with said SLC.

14. The method of claim 13, wherein said IL-2 is recombinant human IL-2.

5

15. The method of claim 13, wherein the route of said administration of IL-2 is selected from the group consisting of: IV, oral, mucosal, intranasal, dermal, i.p., intra-tumor and peritumor routes of administration.

10 16. The method of claim 13, wherein the therapeutically effective amount of IL-2 is approximately 6 million IU/M2 to 600,000 IU/kg.

17. The method of claim 1, further comprising surgical removal of said cancer or hyperproliferative cell growth.

15

18. The method of claim 17, comprising:

- (a) administering said SLC to the site of cancer, tumor, or hyperproliferative cell growth;
- (b) surgically removing of said cancer or hyperproliferative cell growth; and,
- (c) administering said SLC to the site of surgical removal.

20 19. A method for modulating dendritic cell function in a mammal, said method comprising administering a therapeutically effective amount of an SLC agent to said mammal, said agent selected from the group consisting of: SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding SLC polypeptides, variants and fragments, anti-SLC antibodies and ligands for the CCR7 receptor.

20. The method of claim 19, wherein said modulation results in a decreased primary immune response.

CTTGGCAGCTGCCAACCTCACCCCTAGCTCGGCCCTTACTCACCCCTACACAGAC	58
ATGGCTCAGTCAGCTGGCTCTGAGCTCTTATCCTGGTTCTGGCCTTGGCATCCCCAGG	118
M A Q S L A L S L L I L V L A F G I P R	20
ACCCAAAGGCAGTGTAGGGGCTCAGGACTGTGGCTCAAGGTACAGCCAAGGAAGATT	178
T Q G S D G G A Q D C C L K Y S Q R K I	40
CCCCGCCAAGGGTTGTCCGGCAGCTACCGGAGGAACCAAGCTTAGGCTGCTCCATCCCA	238
P A K V V R S Y R K Q E P S L G C S I P	60
GCTATCCCTGTTCTGGCCCGCAAGGGCTCTCAGGGAGGCTATGTGCAAGACCCAAAGGAG	298
A I L F L P R K R S Q A E L C A D P K E	80
CTCTGGGTGCGAGCAGCTGATGGCAGCATCTGGACAAGACACCATCCCCACAGAAACCCAGCC	358
L W V Q Q L M Q H L D K T P S P Q K P A	100
CAGGGCTGCCAGGAAGGACAGGGGGGCTCCAAGACTGGCAAGGAAGGAAGGGCTCCAAA	418
Q G C R K D R G A S K T G K T G S K	120
GGCTGCAAGAGGACTGTAGGGTCAAGAACCCCTAAAGGGCCATAGGCCAGTGGCAGGCCT	478
G C K R T E R S Q T P K G P	134
GGAGCCCTGGAGACCCACCAAGCCTCACCAACGCTTGAAGGCCAAGATGCCAAGA	538
AGGAGGGCTATGCTCAGGGGCCCTGGAGGCCACCCCATGGCTGCCACACTCTTT	598
CTCCTGCTTAACCAACCCCATCTGCATTCAGCTCTACCCCTGCATGGCTGAGCTGCCA	658
CAGCAGGCCAGGTCCAGAGAGACCGAGGGAGAGTCTCCAGGGAGCATGAGAGGGG	718
CAGCAGGACTGTCCCTTGAGGAGAAATCATCAGGACCCCTGACCTGATAACGGCTCCCCA	778
GTACACCCACACCTTCTTGTAAATATGATTATACCTAACTGA <u>ATAAAAGCTGTCT</u>	838
GTCCTCCACCCGCCAAAAAA	864

FIGURE 1

FIGURE 2

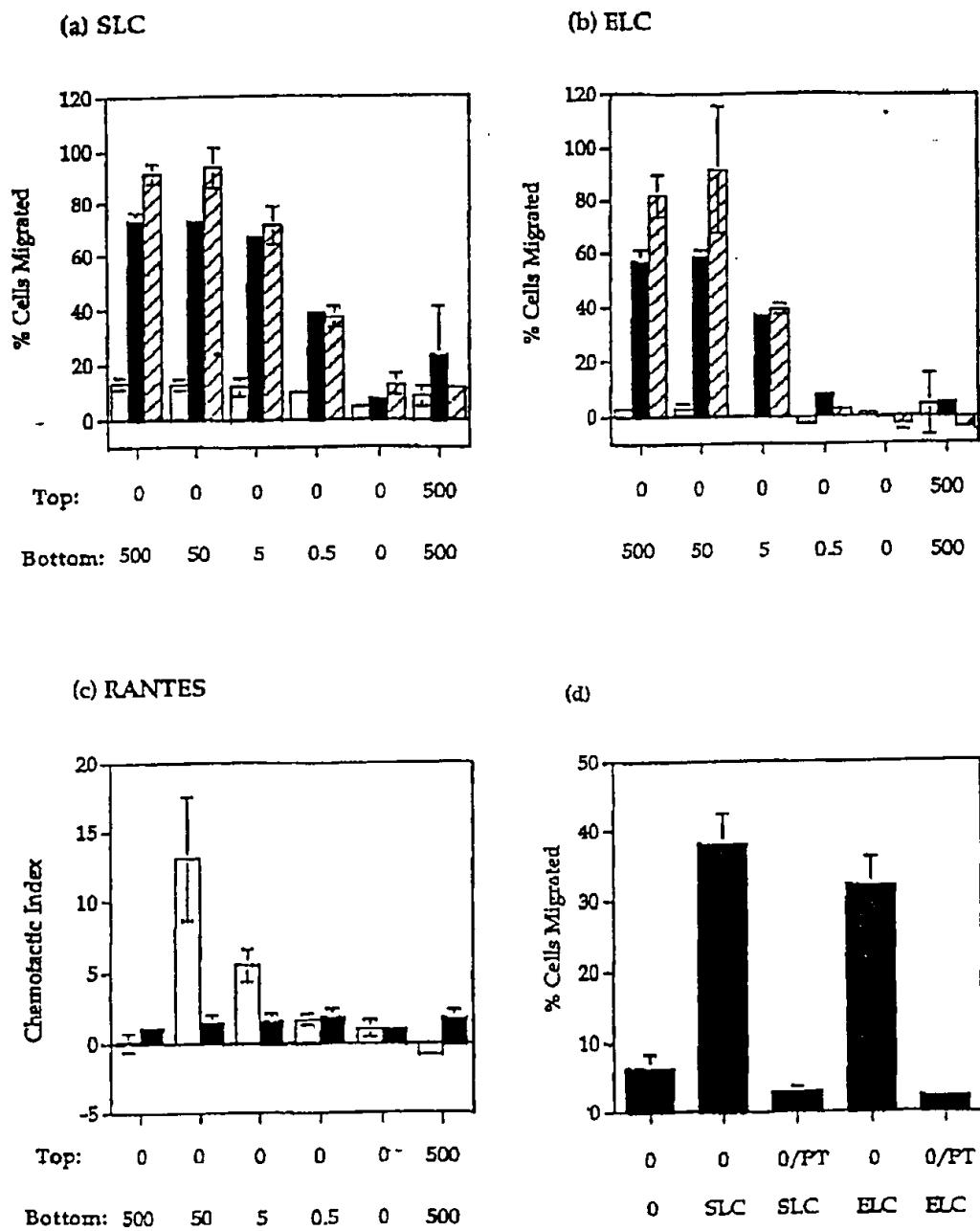


FIGURE 3

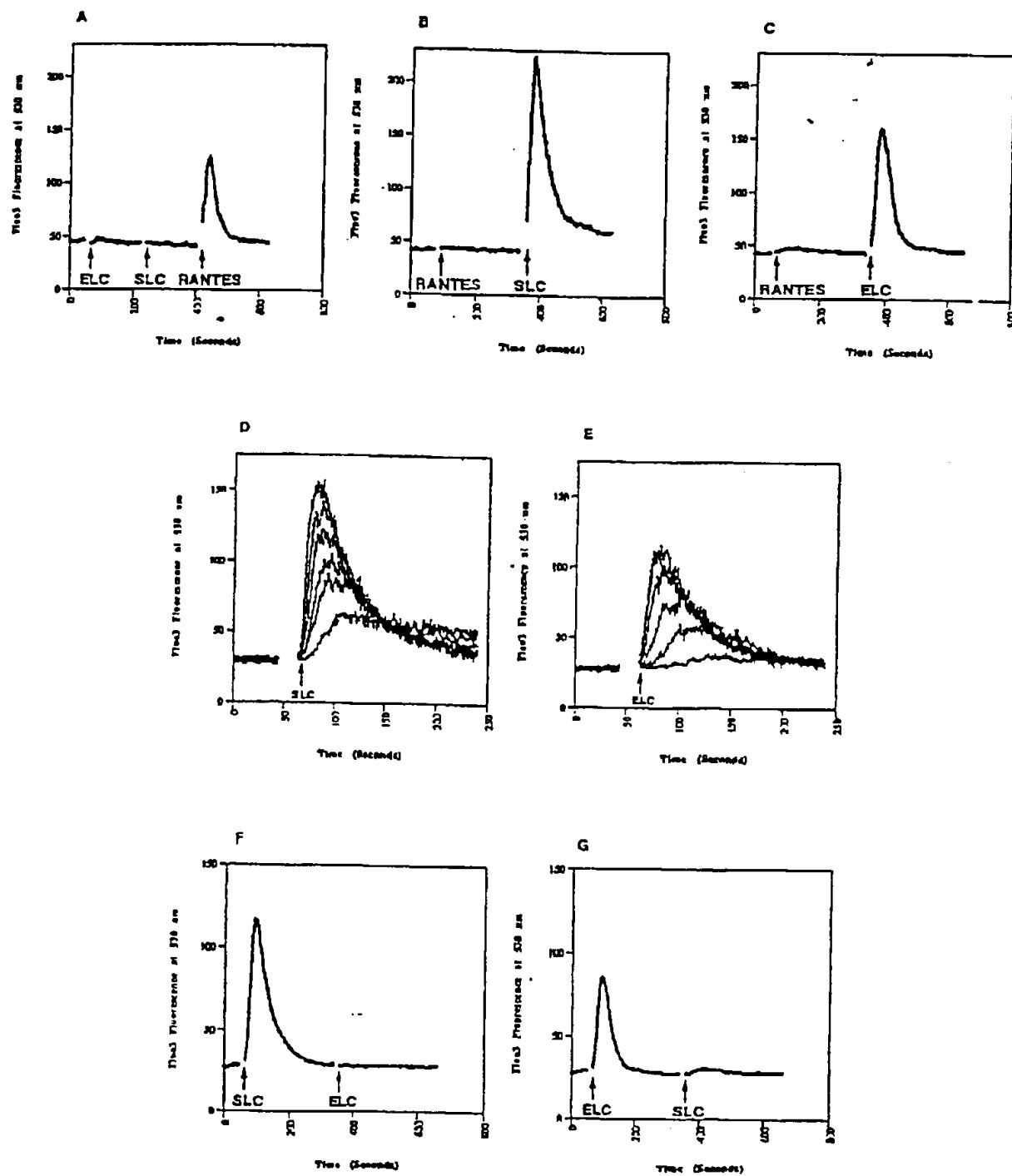


FIGURE 4

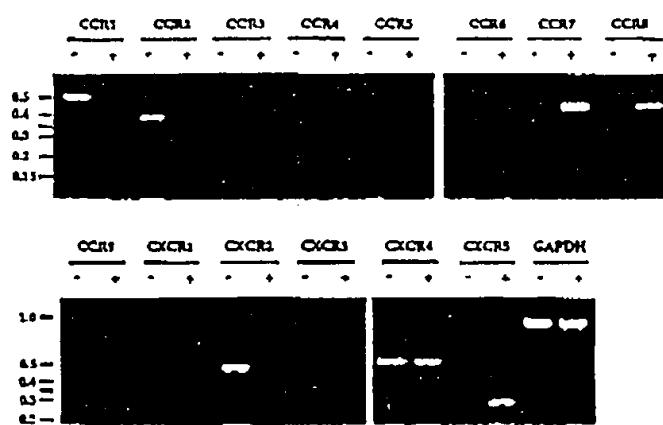


FIGURE 5

Survivors Studies of SLC

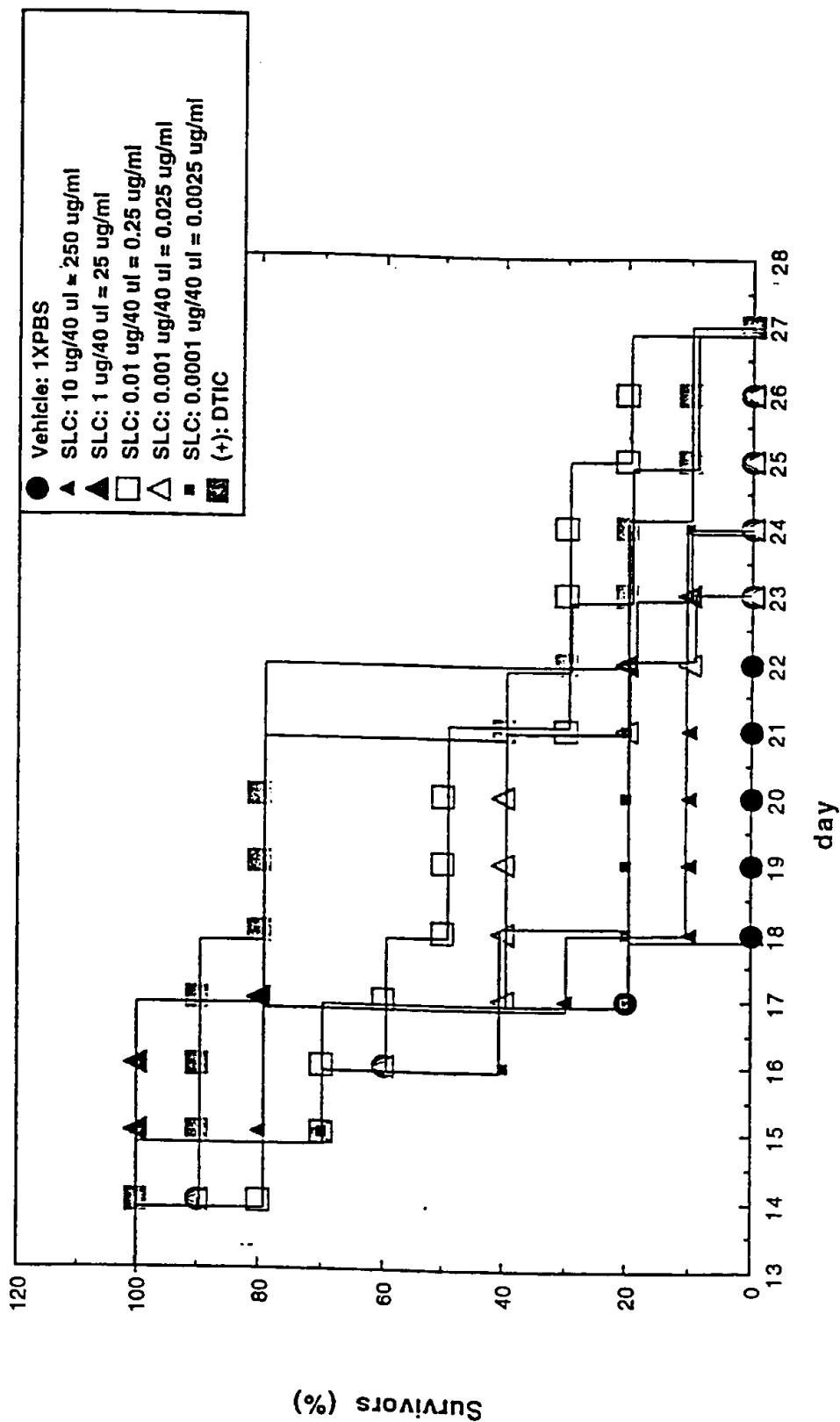


FIGURE 6

Effects of SLC on the Primary Tumor Growth of B16-BL6

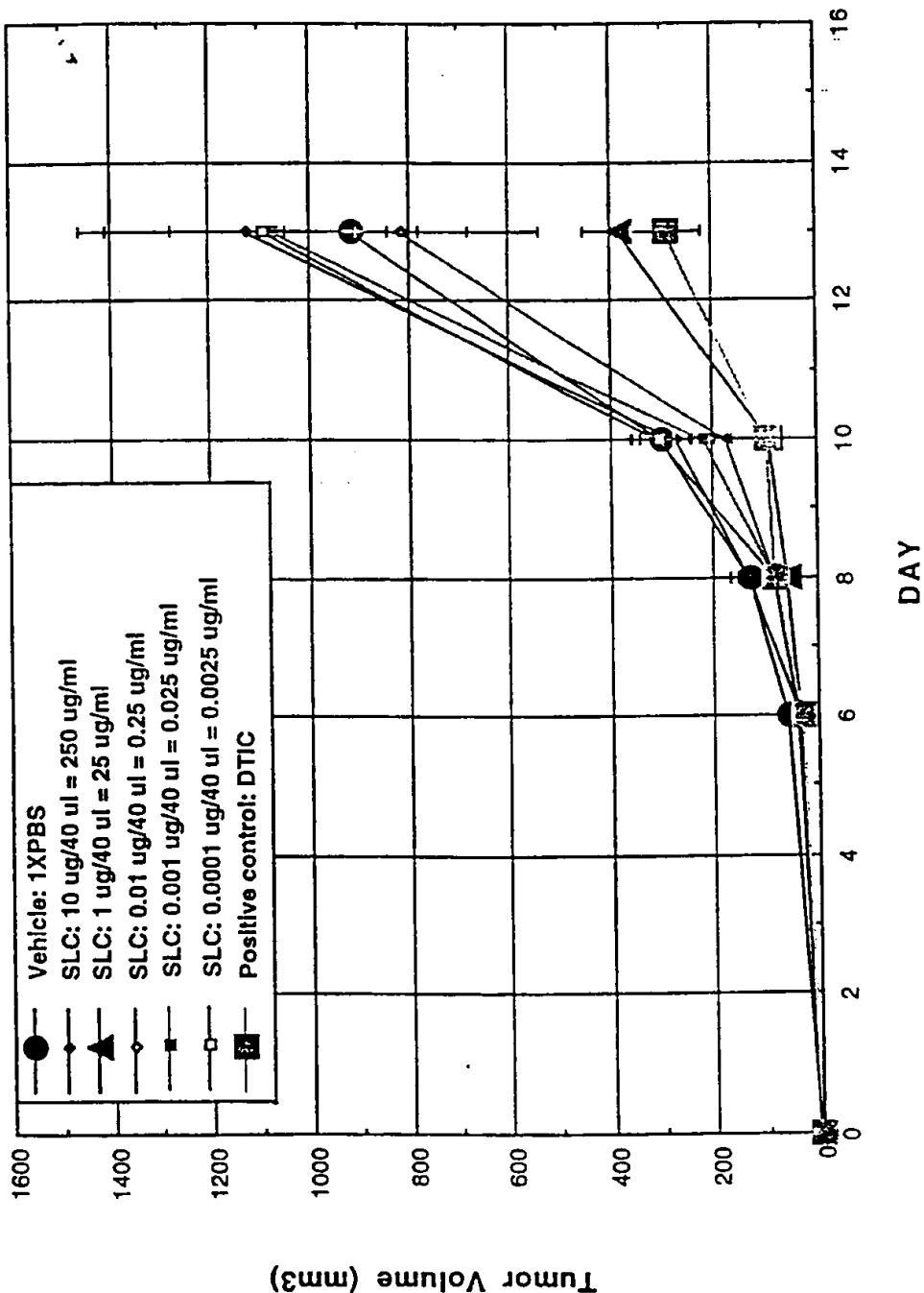


FIGURE 7

Effects of SLC on the Primary Tumor Growth of Day-13

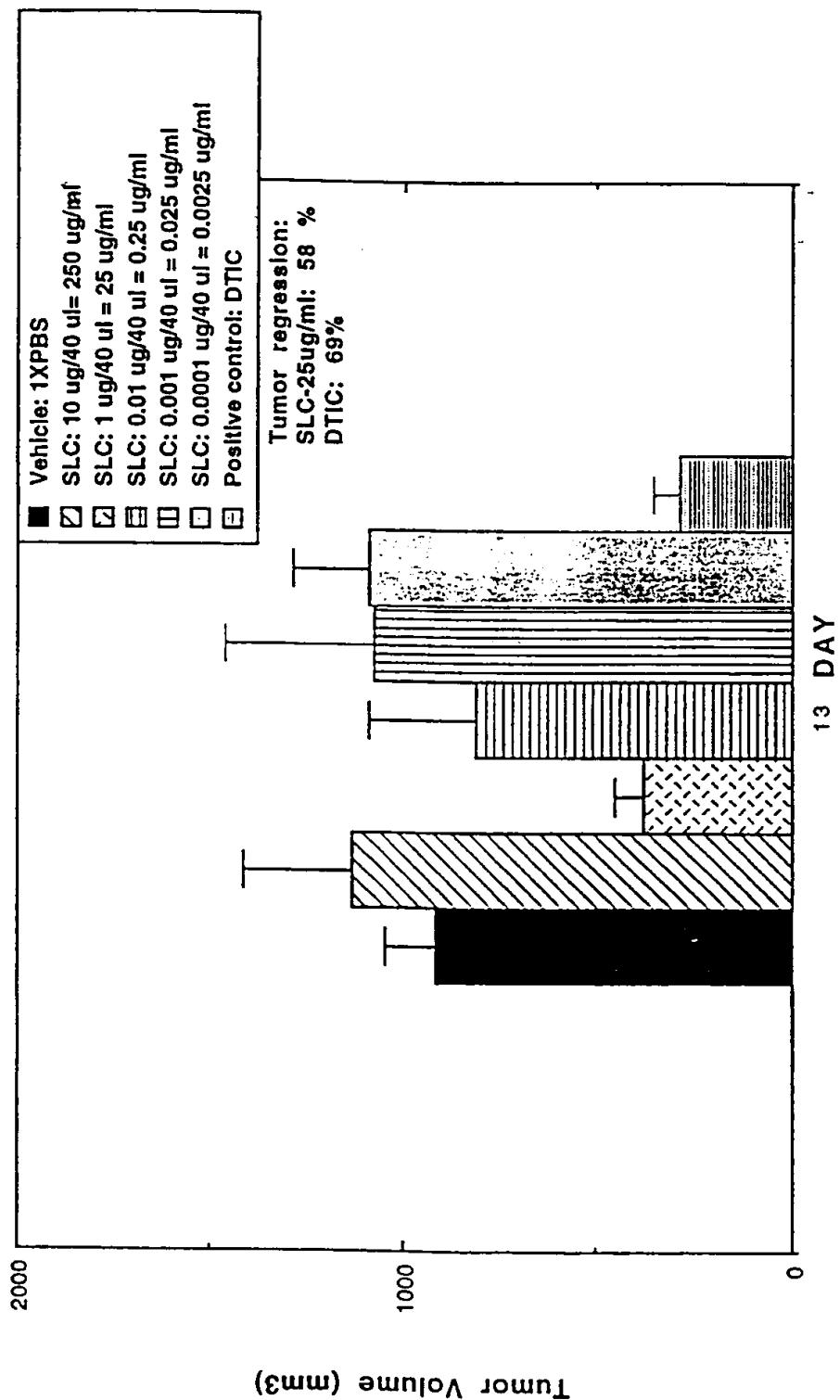
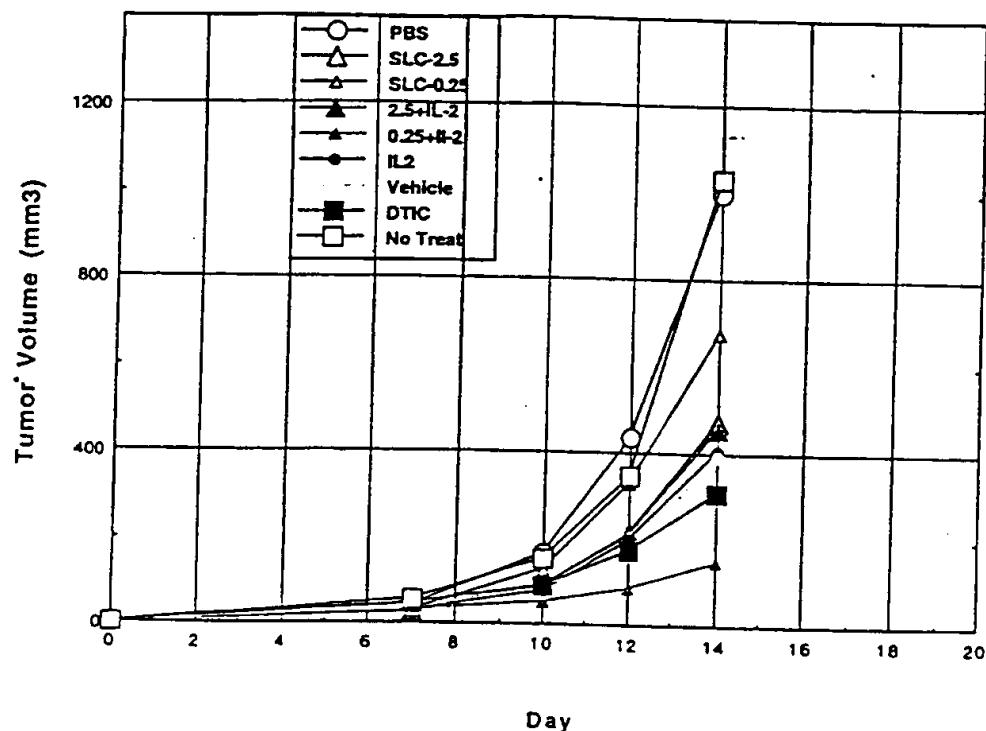


FIGURE 8



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(74) Agents: BLACKBURN, Robert, P.; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608-2916 (US) et al.			
(54) Title: METHODS FOR TREATING CANCER AND FOR MEDIATING CHEMOTAXIS OF DENDRITIC CELLS			
(57) Abstract			
<p>The invention is based on the novel observation, reported herein, that SLC inhibits the growth of tumors <i>in vivo</i> and is a potent chemokine for Dendritic Cells (DC). Thus, the invention provides a method of treating cancer or hyperproliferative disorder in a mammalian subject, comprising: administering a therapeutically effective amount of an SLC to said subject. SLCs useful in the methods of the invention include SLC polypeptides, variants and fragments and related nucleic acids. The methods of the invention are useful in the treatment of cancer and hyperproliferative disorders. In addition, the invention provides methods for modulating dendritic cell function in a mammal, said method comprising administering a therapeutically effective amount of an SLC agent to said mammal, said agent selected from the group consisting of: SLC polypeptides, SLC polypeptide variants, SLC polypeptide fragments, polynucleotides encoding SLC polypeptides, variants and fragments, anti-SLC antibodies and ligands for the CCR7 receptor. Such methods can be used to increase or decrease an immune response, particularly a primary immune response, in a mammal. The methods are particularly useful in the prevention of graft rejection and in the treatment of autoimmune disease. Alternatively, the methods may be used to enhance an immune response.</p>			

**FIGURE 9**

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
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BF	Burkina Faso	GR	Greece			TR	Turkey
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BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
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CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No	PCT/US 99/31096
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A. CLASSIFICATION OF SUBJECT MATTER			
IPC 7 A61K38/19 A61K48/00 A61P35/00 // (A61K38/19, 38:20)			
<p>According to International Patent Classification (IPC) or to both national classification and IPC</p>			
B. FIELDS SEARCHED			
Minimum documentation searched (classification system followed by classification symbols)			
IPC 7 A61K C07K			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)			
EPO-Internal, WPI Data, STRAND, BIOSIS, MEDLINE, CANCERLIT, AIDSLINE, LIFESCIENCES, EMBASE, CHEM ABS Data			
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	
X	SOTO H ET AL: "The CC chemokine 6CKine binds the CXC chemokine receptor CXCR3." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 95, no. 14, 7 July 1998 (1998-07-07), pages 8205-8210, XP002144765 cited in the application the whole document	1-12, 17, 18	
Y	WILLIMANN K ET AL: "The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 28, no. 6, June 1998 (1998-06), pages 2025-2034, XP002121901 cited in the application section "3. Discussion"	13-16	
Y	WILLIMANN K ET AL: "The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 28, no. 6, June 1998 (1998-06), pages 2025-2034, XP002121901 cited in the application section "3. Discussion"	13-16	
-/-			
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.	
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>			
<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"S" document member of the same patent family</p>			
Date of the actual completion of the international search		Date of mailing of the international search report	
11 August 2000		24/08/2000	
Name and mailing address of the ISA		Authorized officer	
European Patent Office, P.B. 5818 Patentien 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Teyssier, B	

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/US 99/31096

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199835 Derwent Publications Ltd., London, GB; Class B04, AN 1998-414107 XP002144766 & WO 98 31809 A (SHIONOGI & CO LTD), 23 July 1998 (1998-07-23) abstract —	1-20
X	WO 96 06169 A (HUMAN GENOME SCIENCES INC ;LI HAODONG (US); ADAMS MARK D (US)) 29 February 1996 (1996-02-29) SEQ ID # 1-2 page 3, line 10 —page 4, line 12	1-12, 17-20
X	WO 98 14581 A (SCHERING CORP) 9 April 1998 (1998-04-09) SEQ ID # 5-8 page 10, line 19 — line 24 page 48, line 33 —page 49, line 30	1-12, 17, 18
E	WO 00 09151 A (SCHERING CORP) 24 February 2000 (2000-02-24) page 1, line 11 — line 16 page 3, line 20 —page 5, line 12	1-12, 17-20

INTERNATIONAL SEARCH REPORT

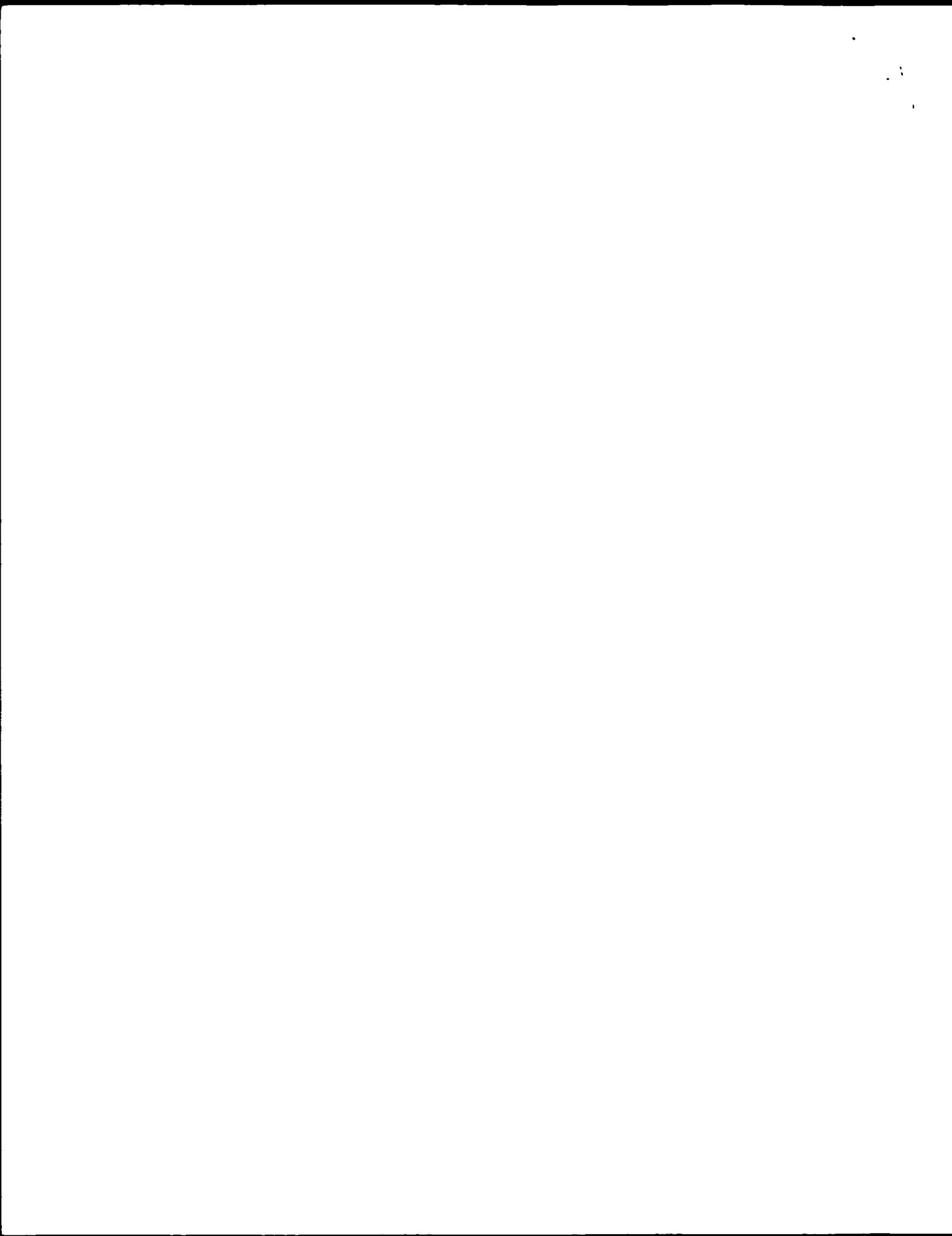
Information on patent family members

International Application No

PCT/US 99/31096

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9831809	A 23-07-1998	AU	5496798 A	07-08-1998
WO 9606169	A 29-02-1996	AU	708558 B	05-08-1999
		AU	2814195 A	14-03-1996
		CA	2198206 A	29-02-1996
		EP	0799311 A	08-10-1997
		JP	10505493 T	02-06-1998
		NZ	288799 A	29-06-1999
WO 9814581	A 09-04-1998	AU	4592397 A	24-04-1998
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DV DNA
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DT 20-OCT-2000
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DR P-PSDB AAB01434
DR WPI 2000-465631/40
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KW SLC
KW Secondary lymphoid chemokine
KW angiogenesis
KW autoimmune disease
KW breast cancer
KW cancer
KW chemotaxis
KW ds
KW graft rejection
KW human
KW hyperproliferative disorders
KW immune response
KW inflammation
KW melanoma
KW metastases
KW proliferative breast disease
KW proliferative retinopathy
KW prostatic hyperplasia
KW suppression
KW tumourigenesis
EKW
OS Homo sapiens.
SC 4a22b9fcfb65eca55a08e9eb4312eae
SP HOMO SAPIENS
ESP
INST (CHIR) CHIRON CORP
CC Secondary lymphod chemokines (SLC's), variants, fragments, and the
CC polynucleotides encoding the chemokines, variants and fragments,
CC anti-SLC antibodies or ligands for the CCR7 receptor can be used to
CC modulate dendritic cell function in a mammal which results in a
CC decreased primary immune response. SLC can be used to treat cancer or
CC hyperproliferative disorders such as prostatic hyperplasia,
CC proliferative breast diseases, proliferative retinopathy or pigmented
CC skin lesions. SLC is also useful for treating solid tumours such as
CC melanoma, breast cancer, tumours of the head and neck, cancers or
CC metastases of ovary, endometrium, urinary tract, stomach, testicle,
CC prostate, lung, bladder, pancreas, bone, liver, colon or rectum, or
CC metastases of unknown primary origin. SLC can also be used to
CC suppress angiogenesis particularly angiogenesis involved in cancer,
CC tumourigenesis, metastases and tumour growth, and for mediating
CC recruitment of leukocytes into sites of inflammation and immune
CC responses, particularly, the chemotaxis of dendritic and other cells.
CC SLC is also useful in preventing graft rejection, prevention and
CC treatment of the autoimmune diseases and for enhancing an immune
CC response.
ECC
RN 1 d5faaac129dfb94c1b4016bb72d762d7
RC PD: 06-JUL-2000. PF: 28-DEC-1999; 99WO-US31096. PR: 31-DEC-1998; 98US-
0114498.
RT Treating cancer or hyperproliferative disorder and modulating dendritic



a mammal involves administering secondary lymphoid chemokine

00038706-A2. Claim 12; Fig 1; 53pp; English.

KETING C

XIN H

CHAN VWF

KOTHAKOTA S

WILLIAMS LT

WINTER JA

463 P 59 463 +

lymphoid chemokine

127 P 59 127 +

460 P 128 460 +

1 BP; 217 A; 279 C; 217 G; 151 T; 0 other;

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Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

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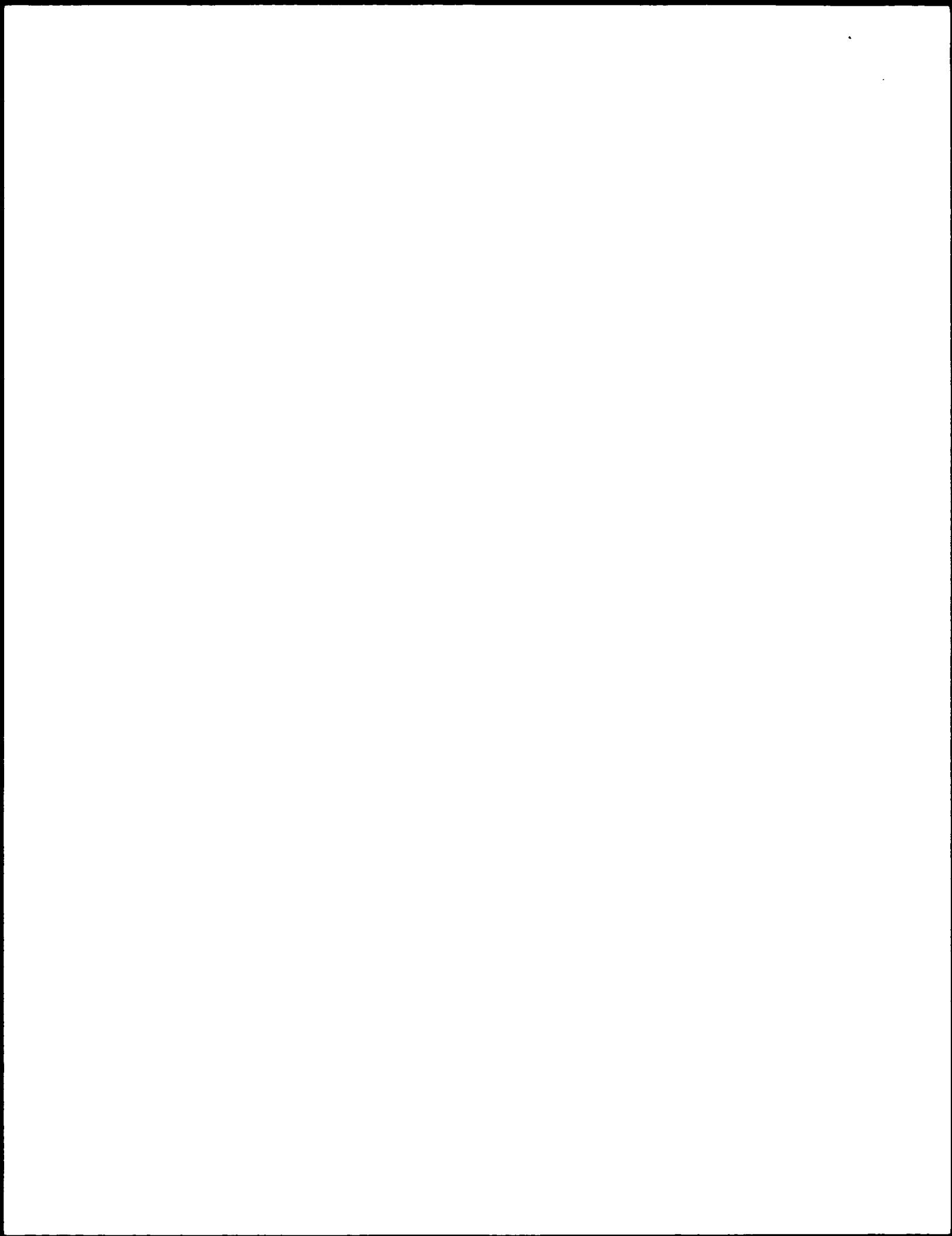
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Identities = 402/402 (100%)
Strand = Plus / Plus

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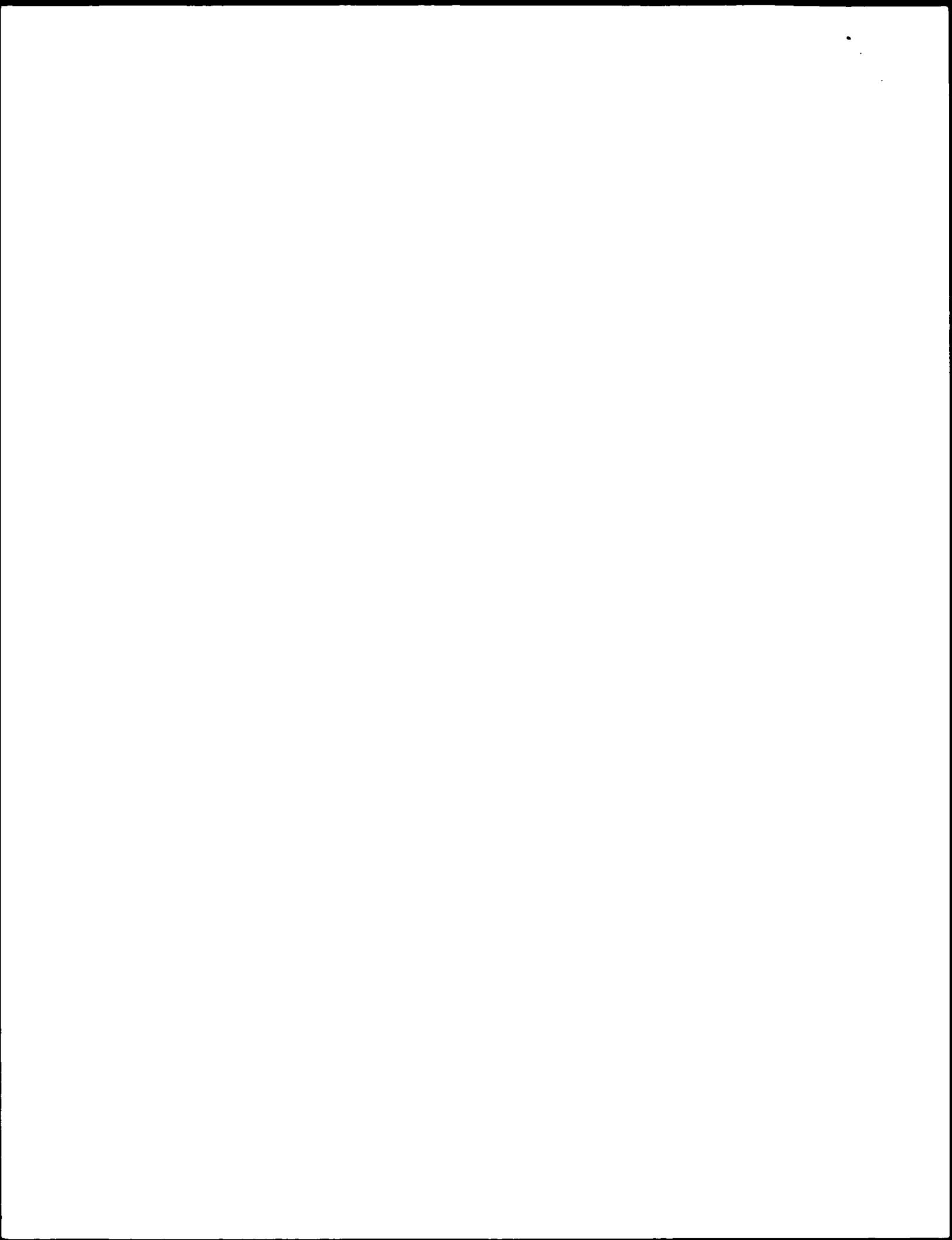
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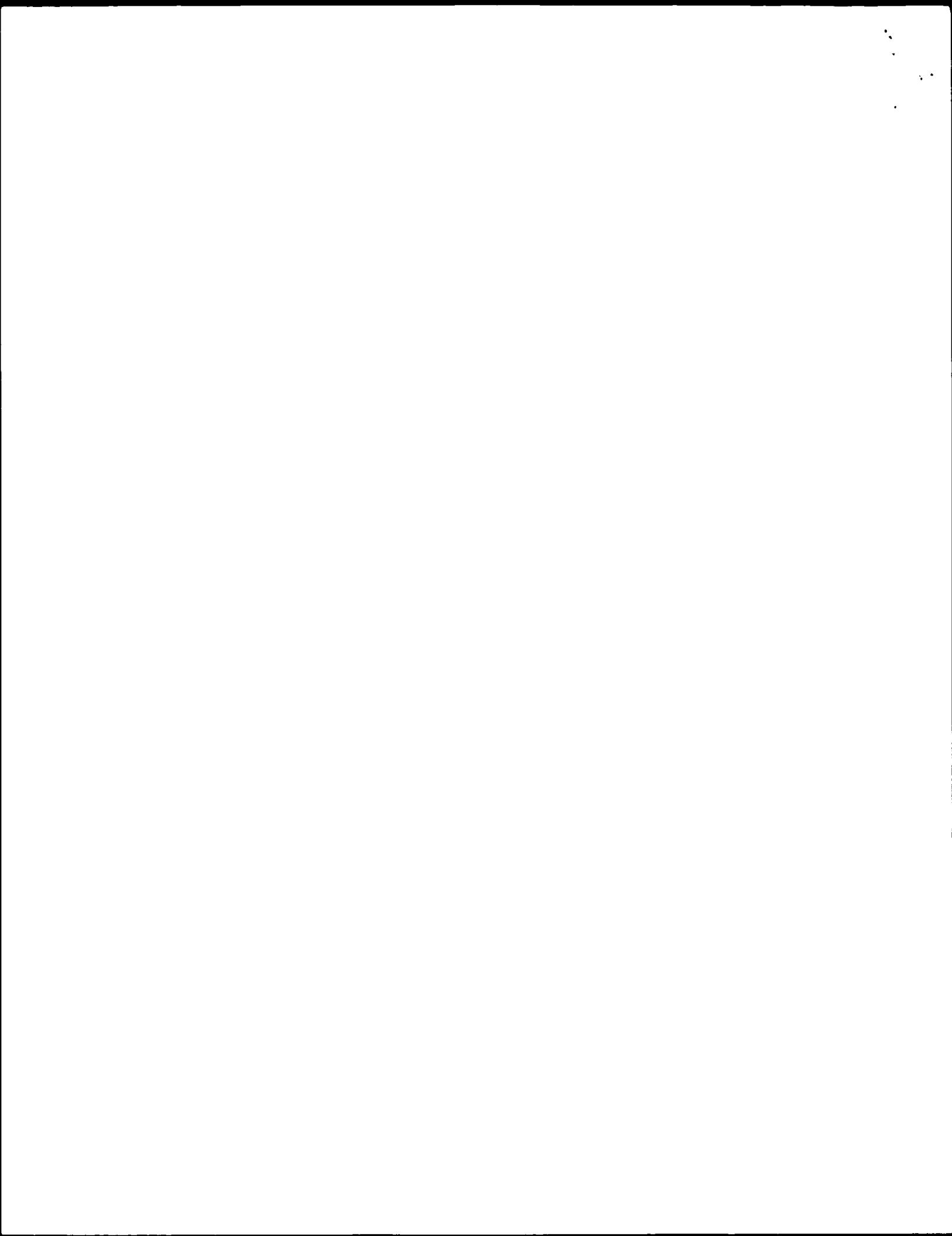
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DV PRT
DT1 20-OCT-2000 (first entry)
DT 20-OCT-2000
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DR WPI 2000-465631/40
EDR
DE Human secondary lymphoid chemokine (SLC).
KW SLC
KW Secondary lymphoid chemokine
KW angiogenesis
KW autoimmune disease
KW breast cancer
KW cancer
KW chemotaxis
KW graft rejection
KW human
KW hyperproliferative disorders
KW immune response
KW inflammation
KW melanoma
KW metastases
KW proliferative breast disease
KW proliferative retinopathy
KW prostatic hyperplasia
KW suppression
KW tumourigenesis
EKW
OS Homo sapiens.
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SP HOMO SAPIENS
ESP
INST (CHIR) CHIRON CORP
CC Secondary lymphod chemokines (SLC's), variants, fragments, and the
CC polynucleotides encoding the chemokines, variants and fragments,
CC anti-SLC antibodies or ligands for the CCR7 receptor can be used to
CC modulate dendritic cell function in a mammal which results in a
CC decreased primary immune response. SLC can be used to treat cancer or
CC hyperproliferative disorders such as prostatic hyperplasia,
CC proliferative breast diseases, proliferative retinopathy or pigmented
CC skin lesions. SLC is also useful for treating solid tumours such as
CC melanoma, breast cancer, tumours of the head and neck, cancers or
CC metastases of ovary, endometrium, urinary tract, stomach, testicle,
CC prostate, lung, bladder, pancreas, bone, liver, colon or rectum, or
CC metastases of unknown primary origin. SLC can also be used to
CC suppress angiogenesis particularly angiogenesis involved in cancer,
CC tumourigenesis, metastases and tumour growth, and for mediating
CC recruitment of leukocytes into sites of inflammation and immune



CC responses, particularly, the chemotaxis of dendritic and other cells.
CC SLC is also useful in preventing graft rejection, prevention and
CC treatment of the autoimmune diseases and for enhancing an immune
CC response.

ECC

RN 1 d5faaac129dfb94c1b4016bb72d762d7

RC PD: 06-JUL-2000. PF: 28-DEC-1999; 99WO-US31096. PR: 31-DEC-1998; 98US-0114498.

RT Treating cancer or hyperproliferative disorder and modulating dendritic cell function in a mammal involves administering secondary lymphoid chemokine to the mammal

RL Patent: WO200038706-A2. Claim 12; Fig 1; 53pp; English.

RA Keting C. KETING C

RA Xin H. XIN H

RA Chan V.W.F. CHAN VWF

RA Kothakota S. KOTHAKOTA S

RA Williams L.T. WILLIAMS LT

RA Winter J.A. WINTER JA

ERN

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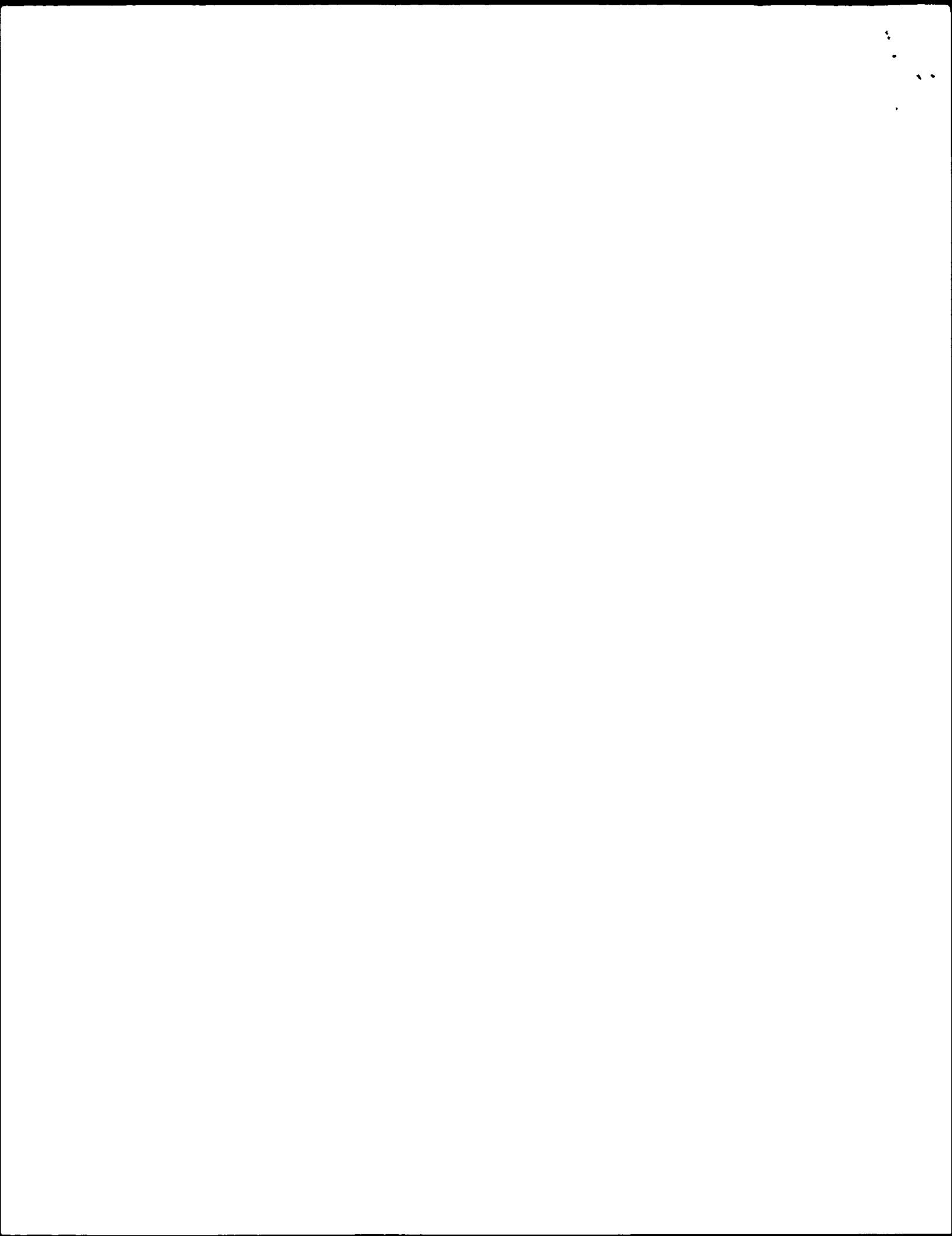
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NCBI-BLASTP 2.0.10 [Aug-26-1999]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

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GSEQ:AAB99211 Murine SLC-interleukin-2 fusion protein.	201	2e-51

>GSEQ:AAB01434 Human secondary lymphoid chemokine (SLC).
Length = 134

Score = 278 bits (703), Expect = 1e-74
Identities = 134/134 (100%), Positives = 134/134 (100%)

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Gapped
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Number of successful extensions: 6373
Number of sequences better than 10.0: 1105
Number of HSP's better than 10.0 without gapping: 438
Number of HSP's successfully gapped in prelim test: 667
Number of HSP's that attempted gapping in prelim test: 5016
Number of HSP's gapped (non-prelim): 1156
length of query: 134
length of database: 130,709,309
effective HSP length: 51
effective length of query: 83
effective length of database: 85,450,940
effective search space: 7092428020
effective search space used: 7092428020
T: 11
A: 40
X1: 16 (7.3 bits)
X2: 38 (14.8 bits)
X3: 64 (24.9 bits)
S1: 41 (21.6 bits)

